

# Fate and Transport of *E. coli* in Rural Texas Landscapes and Streams

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## TSSWCB Project 07-06 Final Report



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## Executive Summary

In September 2006, a seven person Bacteria Total Maximum Daily Load (TMDL) Task Force was charged by the Texas Commission on Environmental Quality and Texas State Soil and Water Conservation Board to evaluate bacteria TMDL development and implementation in the state and develop a list of recommendations for improving this process. Specifically, this Task Force was charged with examining various approaches to develop and implement bacteria TMDLs, recommending economical and timely methods for developing TMDLs and TMDL Implementation Plans (I-Plans), evaluating models and bacteria source tracking (BST) methods available for developing TMDLs and I-Plans and making recommendation for their appropriate use, and with further developing a roadmap for further scientific research needed to reduce uncertainty about bacteria behavior under different water conditions in Texas. These charges and the guidance provided by this Task Force served as the impetus for developing the *Fate and Transport of E. coli in Rural Texas Landscapes and Streams* project.

Within the project, the main objectives are to identify, characterize, and quantify *E. coli* loads resulting from various sources in an impaired watershed; to monitor survival, growth, re-growth, and die-off of *E. coli* under different environmental conditions; to monitor re-suspension of *E. coli* in streams; and educate stakeholders by disseminating qualitative and quantitative information acquired in this monitoring and demonstration project. Information gleaned from this project will provide much needed knowledge relevant to modeling bacteria life cycles, their ability to survive and regenerate, and their impacts on water quality. A secondary objective of this project is to strengthen spatially explicit load allocation tools and validate and improve process-based pathogen transport models used in TMDL development and implementation by providing scientific data collected in this project.

To accomplish these goals and objectives, project personnel conducted four primary tasks: 1) conduct sanitary surveys to identify potential *E. coli* contributing sources in the impaired watershed, 2) conduct demonstration experiments to characterize and quantify *E. coli* loads from identified sources, 3) monitor fate of *E. coli* under different environmental conditions, and 4) monitor concentration of *E. coli* in the instrumented stream. Collectively, these tasks yielded information enabling project staff to identify sources contributing *E. coli* to the selected watershed and stream, to characterize and quantify *E. coli* loads from identified sources, and to clarify *E. coli* fate and transport processes in the watershed. Dissemination of this information to watershed practitioners and stakeholders was also accomplished through the efforts of this project and is critical to the integration of this useful information into future watershed planning and TMDL development efforts across Texas.

Ultimately, project outputs have decreased uncertainties in *E. coli* load estimation from various sources and improved the ability of modelers to simulate the fate and transport processes of *E. coli* in watersheds and streams. Collectively, these outcomes will aid in the development of scientifically sound TMDLs and TMDL I-Plans.

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## List of Abbreviations

Ac	Acre
AgriLife Extension	Texas A&M AgriLife Extension Service
ARS	United State Department of Agriculture, Agricultural Research Service
AU	Animal Unit
BST	Bacterial Source Tracking
CAFOs	Concentrated Animal Feeding Operations
CFUs	Colony Forming Units
<i>E. coli</i>	<i>Escherichia coli</i>
EPA	United States Environmental Protection Agency
FY	Fiscal Year
GIS	Geographic Information Systems
h	hour
MNKA	Minimum Number Known Alive
NAIP	National Agricultural Imagery Program
NLCD	National Land Cover Database
NRCS	United States Department of Agriculture Natural Resources Conservation Service
SELECT	Spatially Explicit Load Enrichment Calculation Tool
TWRI	Texas Water Resources Institute
TDA	Texas Department of Agriculture
TSSWCB	Texas State Soil and Water Conservation Board
TMDL	Total Maximum Daily Load
USDA	United States Department of Agriculture
WPP	Watershed Protection Plan

## Introduction

As of January 2006, 197 water bodies in Texas were considered impaired because they did not meet bacteria criteria established by the state to protect contact recreation (freshwater and saltwater) or oyster water uses. At the time, the freshwater contact recreation use criterion used to determine impairment includes both a geometric mean for indicator bacteria, *Escherichia coli* (*E. coli*), of 126 colony forming units (CFUs) per 100 mL and a single sample maximum of 394 colonies per 100 mL. In 2010, this standard changed to only include the geometric mean of 126 CFUs per 100 mL. Additionally, the number of bacteria impaired water bodies increased to 303 by 2010.

In acknowledgement of increasing numbers of bacteria impairments across the state and the understanding that these issues need to be addressed with a decreasing amounts of resources, the Texas Commission on Environmental Quality (TCEQ) and the Texas State Soil and Water Conservation Board (TSSWCB) convened the Bacteria TMDL Task Force. This Task Force was asked to evaluate current TMDL development processes, address current weaknesses in the process, and to develop a roadmap for further scientific research needed to reduce uncertainty in watershed modeling. As a result of Task Force evaluations, a suite of recommendations for improving the State's response to bacteria impairments were developed. Several of these recommendations included the need to "scientifically" address the current "uncertainties" in bacteria TMDL development and implementation (Jones et al. 2009).

Drawing on these recommendations, the *Fate and Transport of E. coli in Rural Texas Landscapes and Streams* project was developed and funded by the Texas State Soil and Water Conservation Board (Project 07-06). This project was designed to directly address the following recommendations:

- identify and characterize potential *E. coli* sources in an impaired watershed
- monitor fate and transport of *E. coli* from an impaired watershed and stream under varying environmental conditions.
- characterize and quantify *E. coli* loads in identified sources
- monitor *E. coli* concentration changes in response to runoff events and mechanical streambed disturbance

## Source Characterization

As noted in the Bacteria TMDL Task Force Report, high levels of uncertainty often exist in the specification of bacteria source in fate and transport models which lead to further uncertainties associated with the load allocation process in developing a TMDL. This uncertainty starts with the inventory of potential sources of *E. coli* in a watershed and is amplified when pollutant loading estimations are made.

Currently employed methods to identify potential *E. coli* sources in a watershed rely on survey information aggregated at a scale larger than the watershed to quantify these potential sources and typically focus on inputs from human, livestock, and to a limited extent, wildlife. This information is often supplemented by local watershed stakeholders; however, inherent uncertainty in these numbers



still exists. Another common *E. coli* source identification method currently employed is Bacterial Source Tracking (BST).

Each of these methods provides valuable information that can be used to attempt to identify *E. coli* sources, but they cannot quantify the respective loading from a specific source. Sanitary surveys fill this void by inventorying and identifying various potential *E. coli* sources in impaired watersheds. An added benefit to this approach is that it is simpler and less expensive than BST. Sanitary surveys also enable improved population estimates of potential *E. coli* contributors to be determined and allow *E. coli* contributions from each source to be quantified. This results in improved estimation for potential *E. coli* loading within the target watershed.

### Quantifying *E. coli* Loads

*E. coli* load estimation tools such as the Spatially Explicit Load Enrichment Calculation Tool (SELECT) tool are used to estimate *E. coli* loads from various sources and typically rely on literature values for *E. coli* content of feces. Various numbers have been reported in literature for certain domestic and wildlife species and have been summarized in some reports used in TMDL development. However, this information has not been the focus of the reported research and therefore has not undergone extensive peer review. Consequently, there is a high level of uncertainty in identifying *E. coli* loads and sources for use in watershed modeling and *E. coli* load estimation tools.

The deficiency with this approach is that *E. coli* concentrations are not ubiquitous within a species and can vary significantly depending on a variety of factors including diet, climate and even localized population variations. As such, using literature values for *E. coli* content quantified in other regions, states or nations further increases uncertainty in loading estimates. The ability to obtain watershed specific *E. coli* densities will greatly improve accuracy of *E. coli* loading estimates.

Applying a sanitary survey to the target watershed makes this type of data collection possible and provides valuable insight into variation that is exhibited in species specific *E. coli* production. Combined, the accurate identification, characterization, and quantification of *E. coli* sources in the impaired watershed allow for improvements in the predictions of SELECT and other *E. coli* load estimation tools.

### Bacteria Fate and Transport

The Bacteria TMDL Task Force also emphasized the need for additional studies that focus on developing a better understanding of fecal bacteria fate and transport processes. Several aspects of bacteria fate and transport once bacteria are excreted from the host animal are poorly understood. These include fate and transport in rural and agricultural landscapes, re-growth of bacteria in the environment (soil and water) and re-suspension of bacteria in the water column from stream sediments.

Fate and transport of *E. coli* in rural and agricultural landscapes is largely dependent on various environmental factors and management practices. Dominant environmental factors that affect *E. coli* transport in landscapes (e.g., source, soil type, temperature, rainfall, moisture content, nutrient status, etc.) and persistence, re-growth, and survival in landscapes need to be identified. Current knowledge of these processes is limited at best and contributes to significant uncertainties in the modeling of these

processes. Improvements in models require better data on kinetic parameters that describe the fate and transport of bacteria outside the host.

Re-growth of *E. coli* in landscapes is also due to favorable environmental conditions (e.g., rainfall after dry weather conditions) and is another aspect of bacteria fate and transport that is not well understood. The influence of these environmental conditions can individually and collectively impact *E. coli* re-growth and need to be evaluated. These impacts, once identified, will further refine kinetic parameters used in predictive modeling and will improve the representativeness of predicted bacteria loads.

Re-suspension of *E. coli* in streams (e.g. scouring of stream bed sediments due to high flows) is another major fate process that directly influences *E. coli* numbers seen in stream. Unfortunately this process is not well studied or understood. The effect of rainfall and runoff on survival and growth of *E. coli* in streams and stream-bed sediments and subsequent re-suspension of *E. coli* in streams need to be quantified to properly assess the impairment of a stream. Parameters obtained from the stream-monitoring study will be used to improve in-stream hydrodynamic processes modeled by fate and transport models.

Identifying, characterizing, and quantifying *E. coli* loads resulting from various sources are critical tasks in TMDL development for any impaired watershed. Monitoring and assessing the fate and transport processes of *E. coli* in landscapes and streams and monitoring the effects of environmental factors on fate and transport processes are required to develop and validate watershed models that utilize process-based fate and transport subroutines.

## Conducting sanitary surveys to identify potential *E. coli* contributing sources in the impaired watershed

The first Bacteria TMDL Task Force recommendation that this project focuses on is conducting a sanitary survey of an impaired watershed. The Cedar Creek watershed in Brazos and Robertson Counties was chosen for this portion of the project. Cedar Creek was listed as impaired on the 2004 Texas 303(d) List for elevated levels of bacteria (*E. coli*) and does not support its contact recreation standard. The goal of the sanitary survey is to inventory and identify various potential *E. coli* sources that are contributing to the waterbody impairment.

### *Materials and Methods*

To accomplish this goal, the role of livestock and wildlife in *E. coli* transmission in the Cedar Creek watershed was evaluated (Figure 1). Brazos County is located in southeast Texas in the Post Oak Savannah ecotone. Cedar Creek flows southeast for approximately 44 km through Robertson County and the northern part of Brazos County before emptying into the Navasota River on the eastern border of Brazos County. The Navasota River ultimately merges with the Brazos River at the southern tip of the county. Research was conducted on 2 private ranches (Property A, 518 ha; Property B, 660 ha) bisected by Cedar Creek. Each ranch stocked cattle (Property A, 1 cow/10.36 ha; Property B, 1 cow/2.2 ha) on post oak savannah habitat of mixed upland and bottomland grasslands with scattered post oak woodlands located both in the upland and bottomland zones. Both properties exhibited impacts from grazing, though Property B had shorter grasses and more impacted soils likely due to higher cattle stocking rate. Each property had ample available water from Cedar Creek and numerous stock tanks located throughout the properties. Property B had several active oil wells with associated truck traffic and habitat alteration.

Sanitary surveys focused on documenting wildlife were carried out using motion activated, infrared triggered cameras (Non Typical, Inc., Park Falls, Wisconsin, USA) to determine densities of mammals present within the Cedar Creek floodplain (Trolle 2003, Acevedo et al. 2006). On Property A, 30 grid-based points were selected (1 camera/14.3 ha; cameras not allowed on Property B) to place motion operated infrared digital cameras for 25–50 consecutive days once during the winter, summer, and fall seasons (winter, 22 December–21 March; summer, 22 June–21 September; fall, 22 September–December 21) for the 2 year study (Jacobson et al. 1997, Watts et al. 2008). Cameras were placed at observed wildlife trails or openings suitable for camera placement near each pre-determined grid point (Jeganathan et al. 2002, Claridge et al. 2004, Roberts et al. 2005, Trolle and Kéry 2005). Each camera was fitted with a 1 gigabyte flash card capable of storing approximately 1,000 still images and short video clips of approximately 10 seconds. To increase success of camera traps, 2 liters of apple and persimmon-scented gel was applied to substrate (e.g., thick branches, stumps) near the camera every 5th day. Animal densities were determined density using mark-resight methods (Karanth and Nichols 1998, Jacobson et al. 1997, Main and Richardson 2002, Watts et al. 2008). Meso-mammal densities, such as raccoons and opossums, were determined by analyzing trapping numbers in live-trap grids (Main and Richardson 2002). A total of 42 traps were placed on each property using a grid-design. These traps were 81 cm x 25 cm x 30 cm sized for catching raccoons or feral cats (Tomahawk Live Trap,

Tomahawk, Wisconsin, USA) and were placed with 250-m spacing between traps. This density and spacing has been shown to adequately sample animals that are highly attracted to bait (e.g., raccoons, Virginia opossums). Trapping locations were trapped for 12 consecutive days using the Tomahawk box traps baited with canned dog food, apples, bananas, and fish scent. Densities were estimated using mark-recapture methodologies (Krebs 1999). Captured animals were uniquely marked using non-toxic hair dye and released within 5–7 minutes. Sex, age, species, and unique natural marks were recorded. All information was recorded in database and within a Geographical Information System (GIS). Additionally, attempts to trap nine-banded armadillos (*Dasypus novemcinctus*), eastern cottontails (*Sylvilagus floridanus*) and striped skunks (*Mephitis mephitis*) were made on both properties using trap arrays because each species is less attracted or only seasonally attracted to baits. Arrays were fabricated from 61-cm tall poultry netting and 61-cm long wooden stakes. Each array had 8–12 double-door raccoon/rabbit traps (43 traps total for each property; 48 cm x 15 cm x 15 cm; Tomahawk Live Trap, Tomahawk, Wisconsin, USA) with variable array setups designed to take advantage of the local vegetative community and topography.

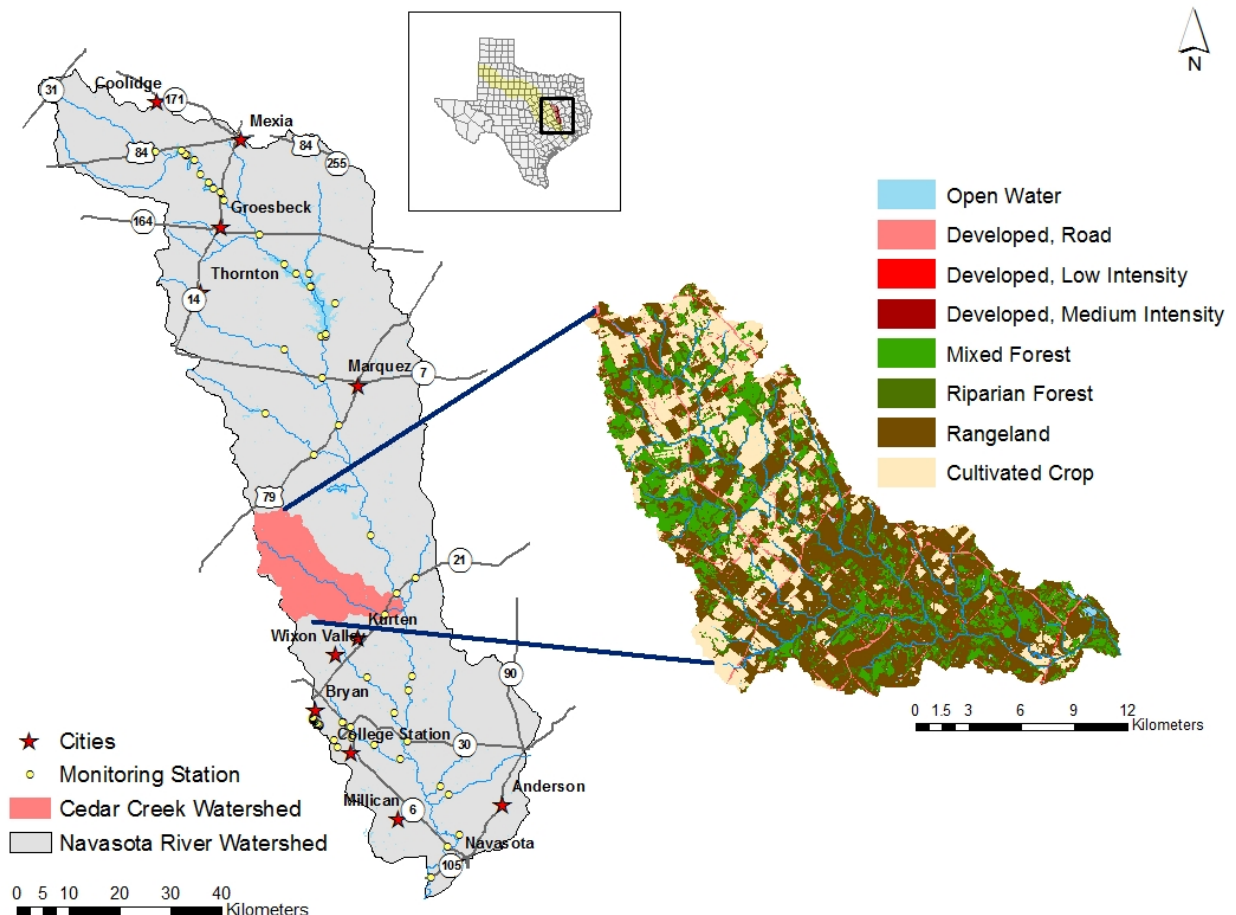


Figure 1. Location of Cedar Creek Watershed in central Texas



Cattle numbers in the watershed were estimated using readily available data. Initially, USDA National Agricultural Statistics Survey data were evaluated; however, these data are aggregated at the county level. Several animal feeding operations were included in these numbers thus skewing the estimated number of cattle upward as no confined animal feeding operations (dairy, feedlot, and poultry farms) were located in the watershed. As a result, USDA NRCS recommended animal stocking rates of 5 acres per animal unit (AU) were used to estimate the total number of cattle in the watershed. This approach was discussed with local resource professionals (NRCS District Conservationist, AgriLife County Extension Agent, property managers) and they concurred that the approach produced a reasonable estimate of cattle in the watershed. Other livestock such as donkeys, horses, goats, sheep and swine were found to be present in nominal quantities.

Additionally, no wastewater treatment facilities are located in the watershed.

### *Population Data Analysis*

Wildlife population estimates were developed using two methods. This approach was chosen as a matter of comparison. First, Schnabel population density tests were conducted on all subject species (Schnabel 1938). The Schnabel estimator is conservative and was the primary estimator for all species densities. Second, capture-recapture data for meso-mammals and white-tailed deer were converted into encounter histories. Program MARK described by White and Burnham (1999), was utilized to develop abundance models. An information-theoretic approach described by Burnham and Anderson (1998) was used to evaluate the null, behavioral, temporal, and requisite interaction models. Model-averaged 95% confidence intervals for all plausible models (Burnham et al. 1987) were calculated. The abundance estimates were compared to estimates reported in the literature and to the Schnabel estimates to ensure validity (Lopez et al. 2003). Finally, in order to compare estimates against conservative numbers, minimum densities for all species based on minimum number known alive (MNKA) from capture histories were generated.

### *Results*

Using the methods described, densities of animal sources identified through the sanitary survey of the watershed were estimated. Of the species identified, estimates were calculated for feral hogs, deer, raccoon, opossum and pastured cattle. Armadillo, cotton-tail rabbit and skunk were also identified, but insufficient data was collected to determine their density.

White-tailed deer data ( $n = 1,025$  total pictures) were gathered concurrently with feral hogs ( $n = 1,487$  total pictures). Grid-trapping was conducted on 2,328 traps-nights during the study (2008–2009) and array trapping totaled 1,680 trap-nights. Although, insufficient numbers of naturally-marked feral hogs were found during any season to conduct model selection approaches; model approaches were successful for raccoons, Virginia opossums, and white-tailed deer. For feral hogs, the sample area was effectively estimated thus allowing conservative density estimation to be made. Schnabel estimators and MNKA were relied upon to calculate conservative population densities for all species (Table 3.1).

Schnabel estimates were comparable to model-selection techniques. Negligible numbers of rabbits, armadillos, and skunks were captured using arrays. Insufficient data was available to calculate feral hog Schnabel estimates for Winter–Spring 2008–2009 and Summer 2009 and Virginia opossum estimates for Summer 2008. Property A had higher densities of raccoons, but Virginia opossums were approximately equal (Table 3.1) on each property.

Table 1. Compilation of density estimates for Property A and Property B, Brazos County, Texas 2008–2009

Property*	Species	Season	Density (km <sup>2</sup> )	CI-Low	CI-High	Minimum density (km <sup>2</sup> ) <sup>a</sup>
<b>A</b>	<b>Raccoon</b>	Summer 2008	88	74	108	49
		Winter 2008	59	46	81	31
		Summer 2009	38	33	45	33
	<b>Virginia Opossum</b>	Summer 2008	12	10	15	12
	<b>White-tailed Deer</b>	Summer 2008	16	14	18	16
		Winter 2008	21	16	29	14
		Summer 2009 <sup>a</sup>	-	-	-	21
	<b>Feral Hog</b>	Summer 2008 <sup>a</sup>	-	-	-	8
		Fall 2008 <sup>a</sup>	-	-	-	5
		Winter-Spring 2008/2009 <sup>a</sup>	-	-	-	5
		Summer 2009 <sup>a</sup>	-	-	-	3
<b>B</b>	<b>Raccoon</b>	Summer 2008	58	47	75	33
		Winter 2008	34	29	42	27
	<b>Virginia Opossum</b>	Summer 2008	11	9	13	11
		Winter 2008	4	3	7	4

<sup>a</sup>Derived from minimum number known alive

\*White-tailed deer and feral hog trapping (physical and camera) were not allowed on Property B

## Conducting demonstration experiments to characterize and quantify *E. coli* loads from identified sources

To minimize the uncertainty of using literature values to estimate the *E. coli* content of feces, work conducted under this task focused on characterizing and quantifying *E. coli* loads resulting from various identified sources.

### Fecal sample collection from relevant and dominant identified sources

#### *Materials and Methods*

Feral hogs were trapped during Summer 2008 (9 traps) and Summer 2009 (6 traps) for 7 days and 6 days respectively using 3-panel corral-style traps on property A. Trapping feral hogs was not allowed on Property B at all and winter trapping was not allowed on Property A due to hunting season. Traps were checked daily and baited with soured corn as necessary during trapping. Hogs were trapped and fecal samples were collected. Sampling on Property A was supplemented by accompanying hog hunters in watersheds near the Brazos River (April 2009).

White-tailed deer were captured during Spring and Summer 2009 using drop nets (Lopez et al. 1998) on Property A. Deer trapping was not allowed on Property B and winter trapping was not allowed on Property A due to hunting season. Drop nets were pre-baited for 7–10 days prior to capture with apple-scented corn. The sex, age and capture location of each animal were recorded using methods described by Lopez et al. (2003). Fecal samples were collected directly from the anus of the immobilized deer.

Fecal material of major contributing species was collected during the summer (2008 and 2009) and winter (2008) seasons (McCleery et al. 2005). Upon animal release, all fecal material from the traps was collected. Traps were cleaned thoroughly using bleach water and scrub brush and the trap was moved to a new location within a 5-m radius to prevent possible cross contamination of subsequent fecal samples (Rutala and Weber 2008). Fresh samples directly from the source animal helped to reduce the risk of environmental contamination of the fecal samples.

All collection and handling of fecal specimens was performed using protective gear (i.e., latex or nitrile gloves). All feces collected were placed in sterile Whirl-Pak containers (Nasco, Fort Atkinson, Wisconsin, USA), were labeled with date of trapping, species information, trap number, tag number (in case of cattle), age and gender of the animal. Age of the animal was broadly categorized by observing the animal into two groups, namely adult and sub-adult. Fecal specimens were placed in an insulated cooler on ice immediately following collection and during transport to the Biological and Agricultural Engineering laboratory at Texas A&M University.

#### *Results*

In total, 362 individual fecal samples were collected from different species during the winter and summer of 2008 and 2009. These data are presented in Table 2 and 3, respectively. Despite this significant number of samples collected, a number of samples were not able to be analyzed due to

sample volume. Fecal samples from meso-mammal species were often too small to produce enough sample to process.

Table 2. Fecal samples from different species collected during the winter

Species	Number of Samples Collected
Cattle	20
Feral hog	24
Raccoon	135
Opossum	73
Skunk	0
Deer	6
Armadillo	2

Table 3. Fecal samples from different species collected during the summer

Species	Number of Samples Collected
Cattle	20
Feral hog	24
Raccoon	135
Opossum	73
Skunk	0
Deer	6
Armadillo	2

### Enumerate *E. coli* and extract *E. coli* isolates from fecal samples

#### *Materials and Methods*

All fecal samples were brought to the laboratory, kept frozen until analyzed. Using a method developed by Byappanahalli et al. (2003), fecal *E. coli* were extracted then enumerated using the USEPA 1603 membrane filtration method (USEPA, 2006). All the samples were analyzed between 24 and 72 hours (h) after they were brought to the laboratory. Fecal samples were first thawed to room temperature. One g of sub-sample was taken from each fecal sample and added to 9.5 mL of sterile de-ionized water in a test tube. Then, the test tube was vortexed for two minutes to elutriate bacteria from the fecal sample. The suspension was serially diluted and filtered using Millipore® 0.45 µm membrane filters. A standard membrane-filtration method (EPA Method 1603) to enumerate *E. coli* in water was used to estimate *E. coli* concentrations. Through this method, vortexed aqueous solution was filtered through a membrane filter placed on a filter base using sterilized forceps to retain the bacteria and then direct count of *E. coli* was obtained based on the development of colonies that grew on the surface of the membrane filter placed on a selective nutrient medium (USEPA, 2002).



The nutrient medium for analyses was prepared by adding 45.6 g of dehydrated modified membrane-Thermotolerant *Escherichia coli* (modified mTEC) agar powder (Becton-Dickinson, NJ) to 1 liter of de-ionized water and then boiling the mixture for one minute. Modified mTEC agar is a selective and differential medium used for chromogenic detection of *E. coli*. The agar was autoclaved at 121°C for 15 minutes, poured into 9 × 50 mm Petri plates, and allowed to solidify at room temperature. Petri plates with membranes were incubated in inverted position for 2 h at 35±0.5°C to resuscitate stressed cells. After 2 h of incubation, Petri plates were transferred into a Whirl-Pak® bag. The bag was sealed and incubated in a water bath at 44.5±0.2°C for 22 to 24 h. The Petri plates were removed from the water bath and the number of red/magenta colonies developed on the membrane were counted and recorded. Aseptic techniques were followed through the experiments and if any growth was observed on a control plate, that counting was rejected. Only the plates having colony counts between 30 and 300 CFUs were used to report *E. coli* concentrations. These counts equated to CFUs per g of wet fecal material. The gravimetric moisture content of all fecal samples were determined simultaneously by drying one g of the wet sample at 100°C for 24 h. Moisture content was calculated on wet basis [(Wet weight of fecal sample – Dry weight of fecal sample) × 100 ÷ Wet weight of fecal sample].

Randomly selected isolates from each plate were streaked on Nutrient agar with, 4-methylumbelliferyl-β-D-glucuronide (MUG), (Difco®) and placed inverted in an incubator at 35±0.5°C for 24 h. MUG is a colorless substrate that is hydrolyzed by an enzyme present in *E. coli*, to a fluorescent product, 4-methylumbelliferone. *E. coli* was confirmed if the MUG plates fluoresced. Confirmed isolates were stored in labeled centrifuge tubes with 1 mL of Luria-Bertani (LB) broth (Difco®) and 10% glycerol in a 20°C freezer.

## Results

*E. coli* concentrations from all species were reported in CFU per g of wet fecal material. Table 4 and 5 present the fecal *E. coli* concentration of the species collected during winter and summer, respectively. All samples were analyzed under similar temperature conditions and collected independent of each other. Different species exhibited considerable variability in the concentration of *E. coli* in their feces. The *E. coli* concentrations from cattle feces were found to be the lowest of all the species analyzed. Cattle feces had the lowest *E. coli* concentration per g of wet sample while deer feces had the highest during winter (Table 4). In summer opossum fecal material had the highest *E. coli* concentration followed by deer feces (Table 5). It was observed that data for all the species were highly skewed with a number of outliers (Padia et al. 2012). A non-parametric analysis of all *E. coli* concentrations of all four species showed a significant difference among the *E. coli* concentrations of the four species ( $p < 0.05$ ). *E. coli* concentrations from feces of different animals were different possibly due to variations in their respective diets. The omnivorous nature of armadillo, opossum, and raccoons could be attributed to higher *E. coli* counts than herbivorous cattle.

Table 4. *E. coli* concentration (CFU/g<sub>wet</sub>) in feces of different species collected during the winter

Species	Number of samples analyzed	CFU/ g of wet fecal material	
		Median	Range
Cattle	2	N/A	$2.81 \times 10^2$ - $9.96 \times 10^2$
Raccoon	35	$5.91 \times 10^4$	$8.95 \times 10^3$ - $1.27 \times 10^7$
Opossum	15	$6.55 \times 10^3$	$9.78 \times 10^1$ - $2.39 \times 10^5$
Skunk	4	$7.83 \times 10^3$	$5.01 \times 10^2$ - $7.62 \times 10^4$
Deer	4	$9.44 \times 10^5$	$2.19 \times 10^5$ - $2.69 \times 10^7$

Table 5. *E. coli* concentration (CFU/g<sub>wet</sub>) in feces of different species collected during the summer

Species	Number of samples analyzed	CFU/ g of wet fecal material	
		Median	Range
Cattle	18	$3.52 \times 10^5$	$3.25 \times 10^3$ - $1.92 \times 10^6$
Raccoon	54	$9.59 \times 10^6$	$9.93 \times 10^3$ - $3.16 \times 10^9$
Opossum	61	$1.45 \times 10^7$	$1.00 \times 10^4$ - $2.78 \times 10^9$
Hog	11	$1.06 \times 10^6$	$7.95 \times 10^4$ - $4.16 \times 10^7$
Deer	6	$4.30 \times 10^5$	$4.60 \times 10^4$ - $1.28 \times 10^6$
Armadillo	5	$1.01 \times 10^7$	$2.95 \times 10^5$ - $4.98 \times 10^8$

## Calculate seasonal *E. coli* loadings from all identified sources

### Materials and Methods

To develop estimated *E. coli* loadings for the entire watershed, data collected must be scaled up to the watershed level. The Spatially Explicit Load Enrichment Calculation Tool (SELECT) model was utilized for this process. SELECT is an automated tool that considers the animal population density, *E. coli* load per animal per day, and suitable land use types for each animal species. The SELECT analysis was conducted at a 30-meter by 30-meter spatial resolution and was completed for both summer and winter scenarios.

For this assessment, an updated land use data set was provided by the Spatial Sciences Laboratory at Texas A&M University. This data was developed using National Agriculture Imagery Program (NAIP) images collected in 2005 paired with 2003 Landsat Satellite Imagery. The land use classification was verified using 2001 National Land Cover Dataset (NLCD) classifications and ground-truthed data. Land use classifications defined for the watershed include open water, roads, low intensity development, medium intensity development, high intensity development, barren land, mixed forest, riparian forest, rangeland, and cultivated land.

Species densities (Tables 6 and 8) were calculated using the respective average winter and summer densities for both Property A and Property B for each species. The cattle density was determined using

the Natural Resources Conservation Service (NRCS) cattle stocking rate for cattle on rangeland for the area. The *E. coli* concentration was calculated using the summer and winter median *E. coli* concentrations respectively. The winter cattle estimate was the exception as the highest concentration in the range was used since there was no median concentration. The *E. coli* load per animal unit per day (Tables 7 and 9) was calculated by multiplying the *E. coli* concentration by the animal's daily fecal production and standardizing it to one animal unit (1,000 lbs of animal weight). The daily fecal production was determined as one percent of the animal's body weight. Each source was distributed to suitable areas in the watershed for both seasons using the densities in Table 6 and 8. Then the density was multiplied by the *E. coli* load per animal. Raccoons and opossums were applied to riparian and mixed forest areas within a 100 meter riparian buffer around the watershed hydrography. Suitable areas for cattle were rangeland, riparian forest, and mixed forest. Feral hogs were distributed around a 100 meter riparian buffer within all land use types except urban areas. Deer were applied to areas with at least 20 acres of contiguous habitat within riparian and mixed forest lands. After the potential *E. coli* loads were calculated, the results were aggregated at the sub-watershed level by the SELECT model to easily distinguish areas of concern.

Table 6. Average animal density applied in SELECT of different species during the winter

Species	Density (animal/km2)
Raccoon	46.5
Opossum	4
Deer	21
Cattle	49.4

Table 7. *E. coli* load (CFU/AU/day) applied in SELECT of different species during the winter

Species	<i>E. coli</i> Density (CFU/g)	Fecal production (kg/AU/day)	<i>E. coli</i> Load (CFU/AU/day)
Cattle *	9.96E+02	18.14	1.81E+07
Raccoon **	5.91E+04	0.11	6.50E+06
Opossum **	6.55E+03	0.03	1.83E+05
Deer **	9.44E+05	6.8	6.42E+09

\* high value is used

\*\* median value is used

Table 8. Average animal density applied in SELECT of different species during the summer

Species	Density (animal/km <sup>2</sup> )
<b>Raccoon</b>	61.3
<b>Opossum</b>	11.5
<b>Deer</b>	16
<b>Feral Hog</b>	5.5
<b>Cattle</b>	49.4

Table 9. *E. coli* load (CFU/AU/day) applied in SELECT of different species during the summer

Species	<i>E.coli</i> Density (CFU/g)	Fecal production (kg/AU/day)	<i>E.coli</i> Load (CFU/AU/day)
<b>Cattle</b>	3.52E+05	18.14	6.39E+09
<b>Raccoon</b>	9.59E+06	0.11	1.05E+09
<b>Opossum</b>	1.45E+07	0.03	4.06E+08
<b>Feral Hog</b>	1.06E+06	4.54	4.81E+09
<b>Deer</b>	4.30E+05	6.8	2.93E+09

### Results:

The SELECT model presents a total potential loading estimate for each species and aggregates it to subwatersheds within the larger watershed. This approach yields directly comparable results within an individual species and clearly illustrates what subwatershed(s) within the modeled watershed have the highest potential *E. coli* from the modeled species. Comparisons between species can also be made, but the scale of potential *E. coli* loading estimates must be carefully considered as there is often several orders of magnitude difference between species.

Figures 2 through 10 illustrate the total potential *E. coli* loads calculated for the watershed in the winter and summer using the respective animal densities and *E. coli* production rates of the modeled species during each respective season.



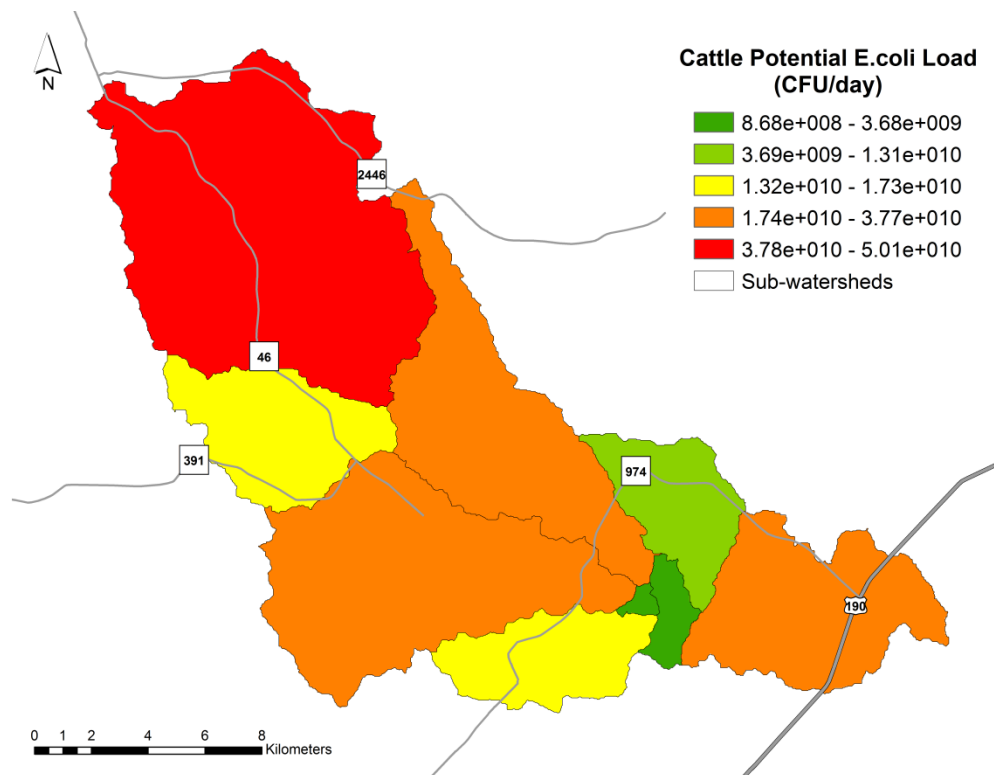


Figure 2. Daily potential *E. coli* load resulting from cattle during the winter

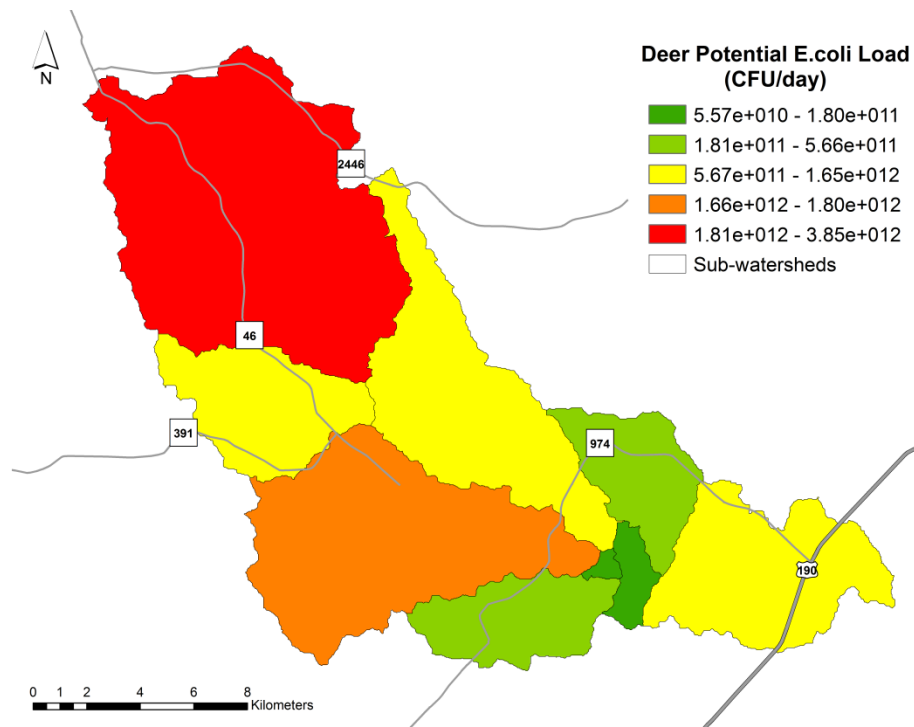


Figure 3. Daily potential *E. coli* load resulting from deer during the winter

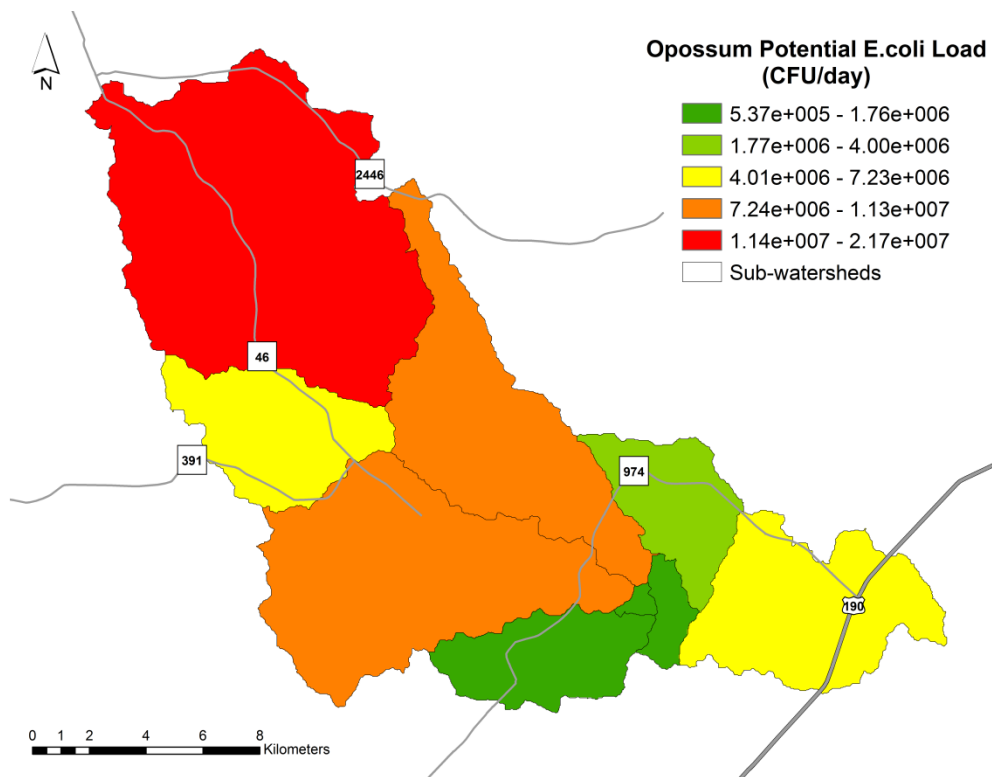


Figure 4. Daily potential *E. coli* load resulting from opossum during the winter

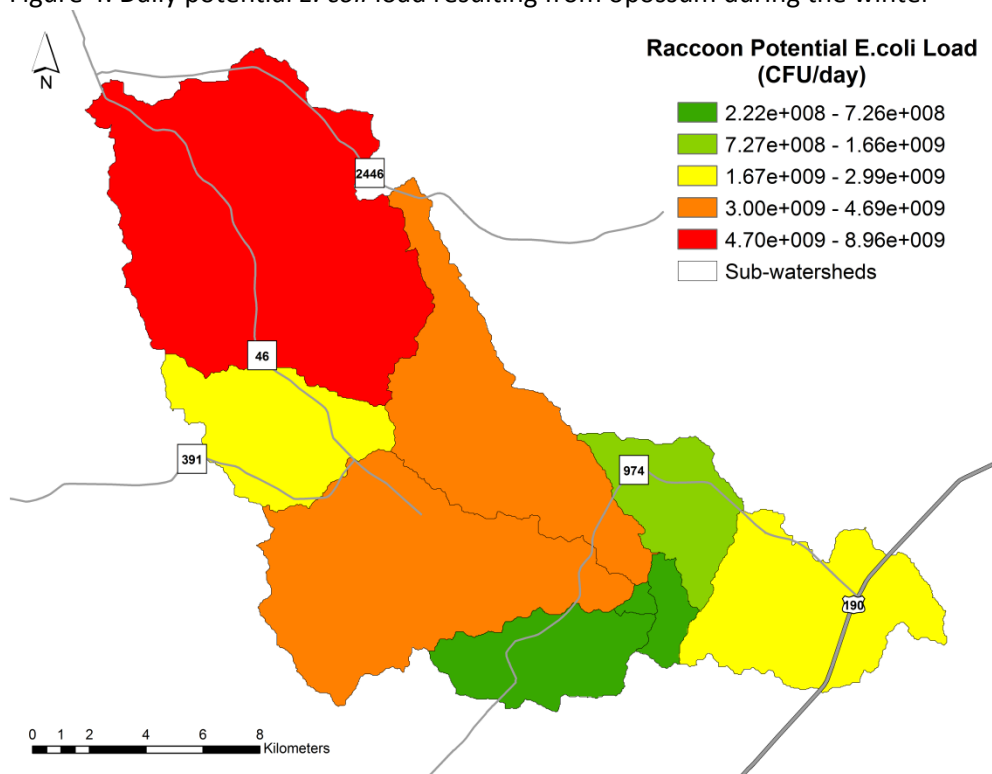


Figure 5. Daily potential *E. coli* load resulting from raccoon during the winter

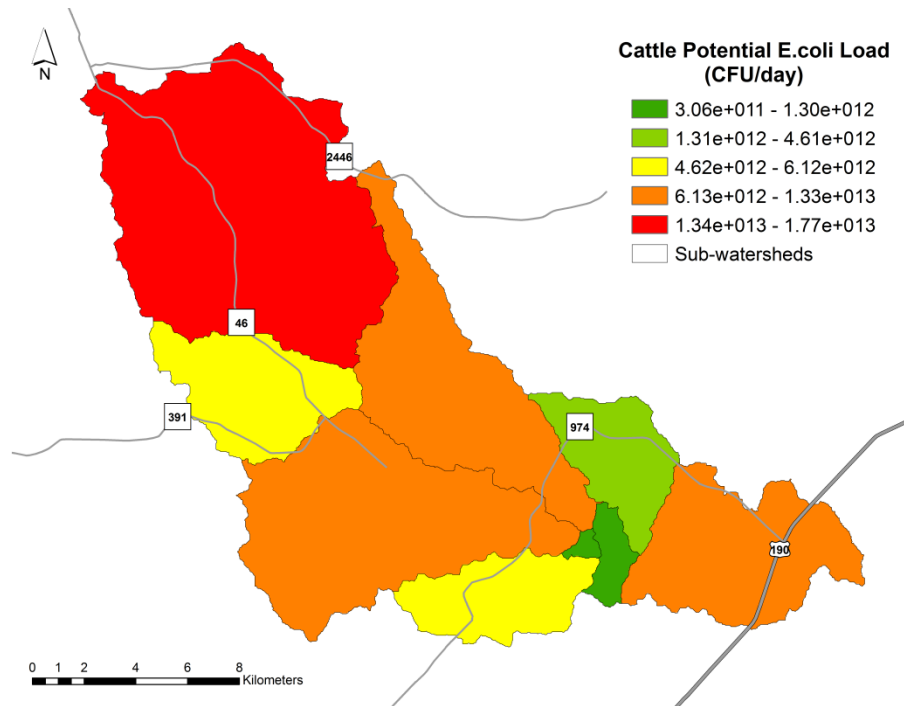


Figure 6. Daily potential *E. coli* load resulting from cattle during the summer

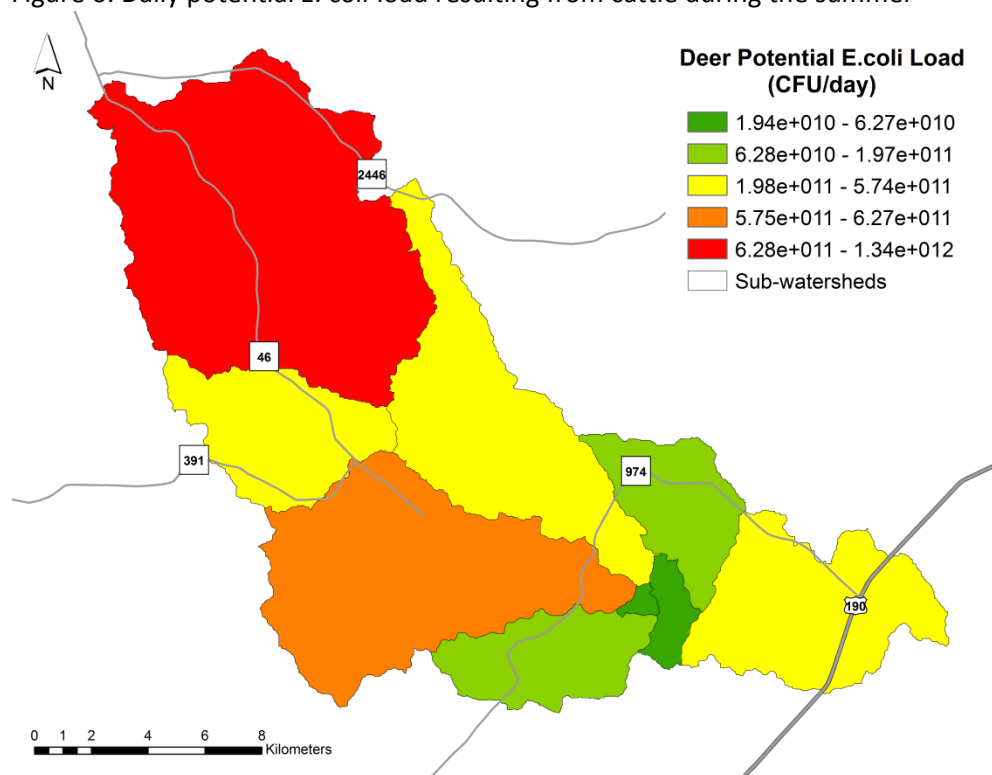


Figure 7. Daily potential *E. coli* load resulting from deer during the summer

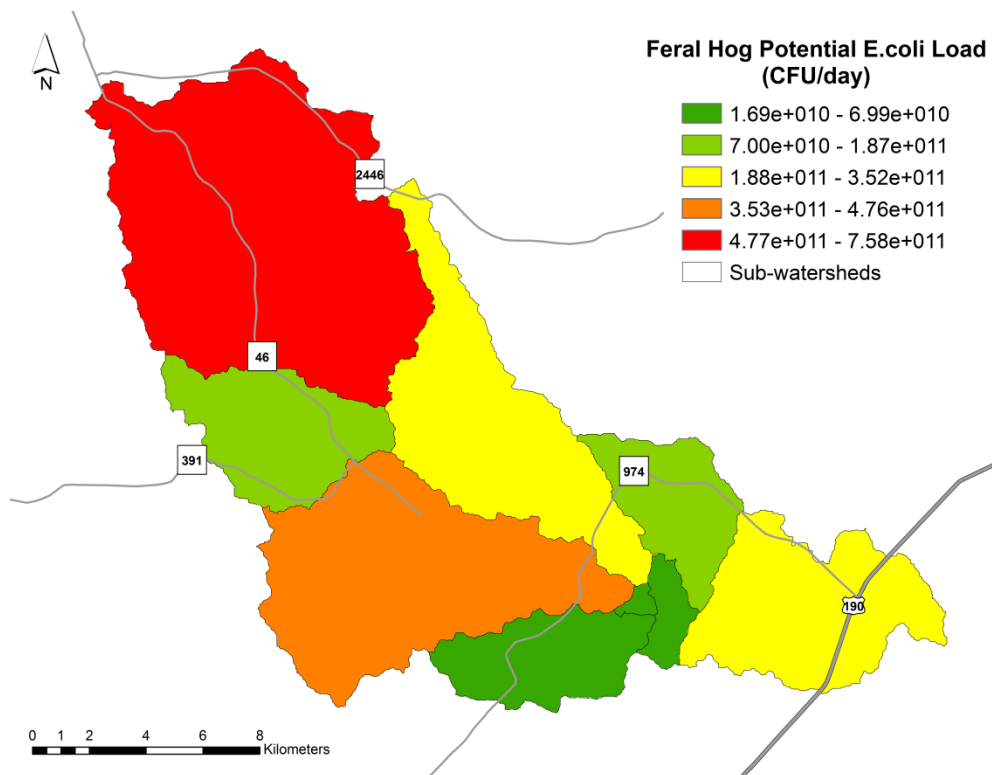


Figure 8. Daily potential *E. coli* load resulting from feral hog during the summer

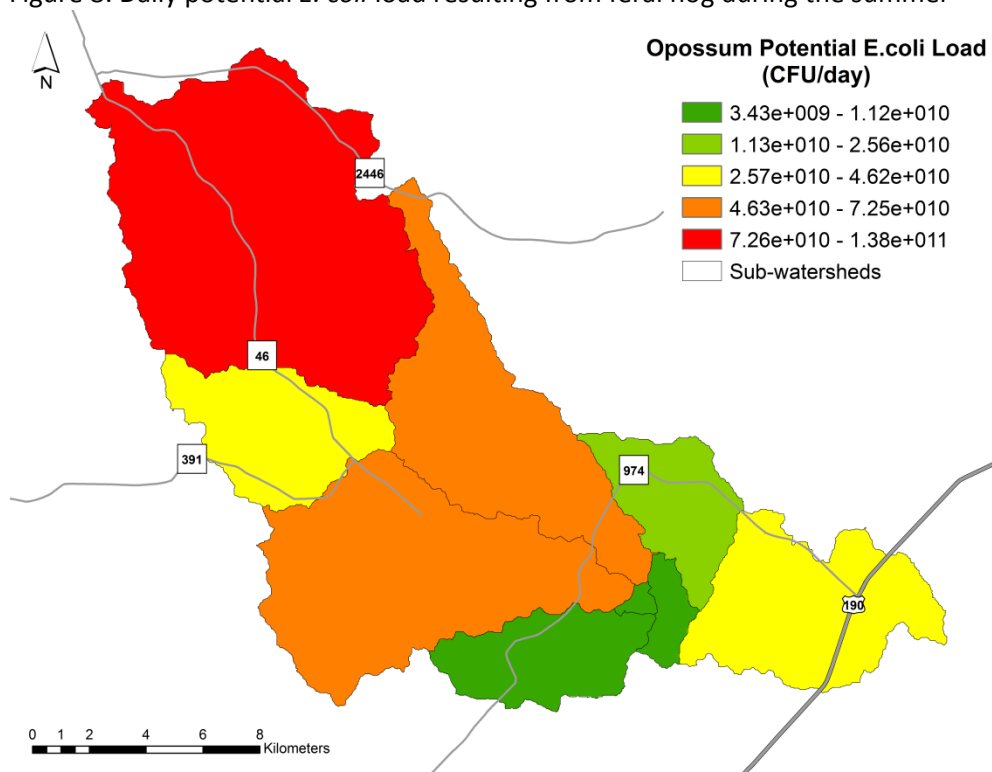


Figure 9. Daily potential *E. coli* load resulting from opossum during the summer



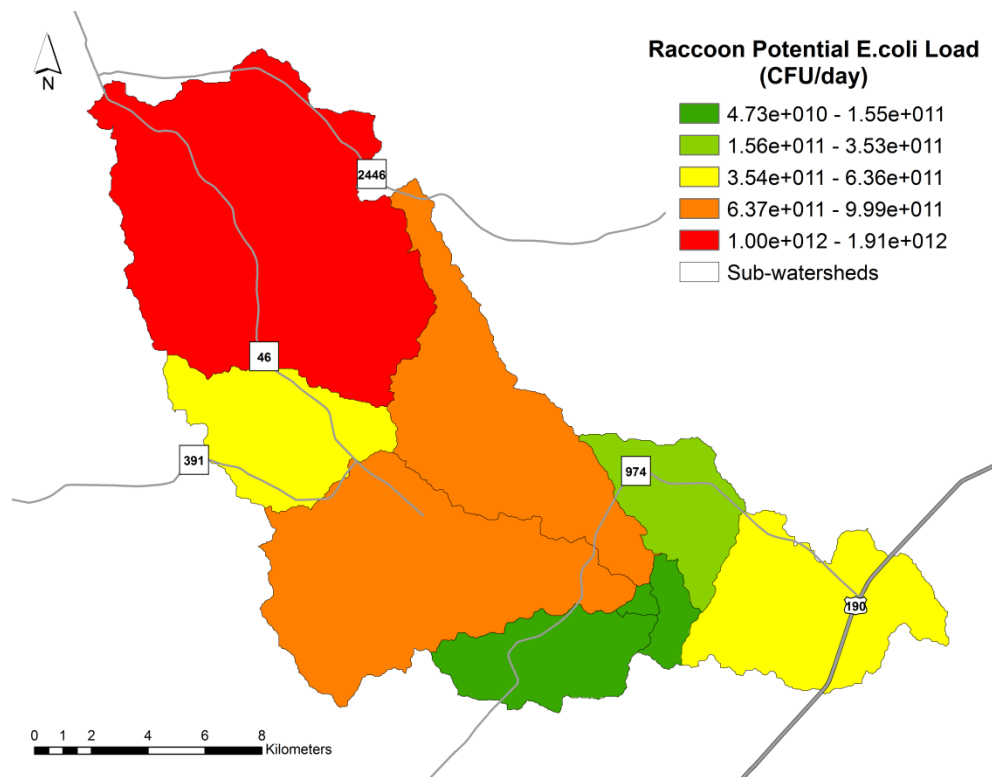


Figure 10. Daily potential *E. coli* load resulting from raccoon during the summer

### Discussion

In summer, opossum fecal material had the highest median *E. coli* concentration of  $1.45 \times 10^7$  CFU/g<sub>wet</sub> and cattle fecal material had the lowest median *E. coli* concentration of  $3.52 \times 10^5$  CFU/g<sub>wet</sub> (Table 9). During winter (Table 4), the highest median *E. coli* concentration was from deer fecal material ( $9.44 \times 10^5$  CFU/g<sub>wet</sub>) and the lowest median *E. coli* concentration was from opossum fecal material ( $6.55 \times 10^3$  CFU/g<sub>wet</sub>). Median *E. coli* concentrations from deer fecal samples collected in the summer and winter (Table 7) were not significantly different ( $p < 0.05$ ). However, median *E. coli* concentrations were significantly higher for both raccoon and opossum fecal samples collected during summer than winter ( $p < 0.05$ ).

In general *E. coli* concentrations in feces of different species varied and season (*summer and winter*) played a significant role in the variability as well. For more discussion on the effects of age and gender, please refer to the published manuscripts included in Appendix A and B.

Interpretation of SELECT model results requires evaluating the scale of the model outputs as well as their distribution across the modeled watershed. When comparing model outputs within and between species, the graphic outputs often look identical or at least similar. Despite these similarities in color depicted, the estimated loading that is allocated to each color can be quite different. As a result, it is critical that the range of *E. coli* loads allocated to each color be evaluated for each output.

Additionally, *E. coli* loads are aggregated to the sub-watershed level. Both the size of the sub-watershed and the amount of suitable habitat area within the sub-watershed impacts the amount of potential *E. coli* load aggregated to a particular sub-watershed. A larger sub-watershed generally has more suitable habitat area to aggregate within that sub-watershed and therefore, generally has more potential *E. coli* load to aggregate as compared to a smaller sub-watershed.

Across all of the potential sources for both summer and winter months, the largest sub-watershed in the watershed has the highest potential load and the smallest sub-watersheds have the lowest potential load. Since the sources were distributed on the same suitable areas regardless of season, the graphics look identical for the same source for both seasons with the only change being the actual amounts of potential *E. coli* load. This also applies to the similarities between the opossum and raccoon outputs because both sources were applied to the same suitable areas.

## Monitoring fate of *E. coli* under different environmental conditions

This portion of the project focused on identifying and improving knowledge of the influences that dominant environmental factors have on *E. coli* fate and transport. To evaluate these influences, a series of studies were carried out to quantify the survival and regrowth potential of *E. coli* isolated from each evaluated species.

### Survival of *E. coli* from different sources under varying environmental conditions

#### Materials and Methods

##### Study 1

Ten g of fecal sample (*cattle and raccoon*) were mixed with 95 mL of sterilized water from Cedar Creek (autoclaved three times at 121°C for 15 minutes). The mixture was then divided into four equal volumes in sterilized bottles and stored at 0°C, 10°C, 25°C and 50°C. *E. coli* in water were enumerated after 1, 24, 72, 120, and 168 h using EPA Method 1603. Enumerations were done in triplicate for each monitoring time and the median of the three *E. coli* enumerations was reported as CFU per 100 mL.

##### Study 2

Three *E. coli* isolates from fecal samples of two wildlife species (*hog and deer*) were enriched in 100 mL of Luria-Bertani (LB) broth at 35.5°C for 24 h. The enriched LB broth was diluted to 10<sup>4</sup> by adding 1 mL of LB broth into 100 mL sterile DI water. This mixture was stirred, then 1 mL was added to 100 mL of sterile DI water. Finally, creek water microcosms were made with 1 mL of the 10<sup>4</sup> dilution of LB broth in 100 mL of sterile Cedar Creek water (autoclaved three times at 121°C for 15 minutes). Microcosms were triplicated and incubated at different temperatures (10, 25, and 30°C) for 30 h. At each sampling time, 0.1 mL of each water microcosm was spread plated onto MacConkey agar (Difco) plate at six different sampling times. Triplicate *E. coli* bacterial counts (CFU/mL) were recorded at six different time samples at each temperature (10, 25, and 30°C).

##### Study 3

Isolates of the same fecal samples used in studies 1 and 2 were used to study the survival of *E. coli* at different moisture contents in soil. *E. coli* isolates were streaked on LB agar and allowed to grow for 24 h at 35°C. Of the colonies obtained after 24 h, one randomly selected colony was cultured in LB broth at 35°C for 24 h. A sterilized bottle was filled with 30 g of sterilized (autoclaved three times at 121°C for 15 minutes) soil from Cedar Creek and 1 mL of the inoculated broth was added to the soil in each bottle. To evaluate *E. coli* response to soil moisture, 0, 6, 15, and 22.5 mL of sterile DI water was added to the soil with inoculum to obtain 4%, 25%, 56.5% and 83% moisture contents, respectively. Soil samples were incubated at room temperature. *E. coli* in soil was enumerated after 1, 24, 72, 120, and 168 h. Enumerations at each sampling were done in triplicate and the median *E. coli* numbers was reported as CFU per g dry weight of soil. Growth and die-off at above mentioned environmental conditions was monitored. Growth or decay rates were determined by plotting *E. coli* concentration with respect to time.

## Results

Generally, the results of *E. coli* analysis at different temperature and moisture conditions monitored over a 7 day period showed considerable variability in *E. coli* response and exhibited different trends in their fate. Figures 11 through 14 illustrate median *E. coli* concentrations quantified per 100 mL of water or g of dry soil and demonstrate the observed differences in variability. The observed declines on any given day might be due to the depletion of nutrients over time and increased competition for nutrients within bacterial population. Observed re-growth could possibly have occurred due to the nutrition available from the organic matter of the dead bacterial cells.

### *Effects of Temperature on E. coli Survival in Water*

The survival of *E. coli* from cattle and raccoon in water at different temperatures over a 7 day period showed highly variable *E. coli* counts (Figures 11 and 12). *E. coli* concentrations in cattle and raccoon fecal samples at the beginning of the experiments were determined after one hour. These background concentrations are presented for comparison with the bacterial concentrations from subsequent days. For both species maximum survival and growth of *E. coli* was observed at 20°C and no growth was seen at 50°C.

At 0°C, there is a slight decrease in cattle *E. coli* growth after 24 h. The concentration increased after 72 h and then decreased until the end of the incubation period (Figure 11). *E. coli* from raccoon feces at 0°C (Figure 12) showed a decrease after 24 h, increased at 72 h, decreased at 120 h, and increased again after 168 h.

A gradual increase in the cattle *E. coli* concentration was observed at 10°C until the 120 h and then decline by one order of magnitude after 168 h (Figure 11). Similar to the response at 0°C, *E. coli* from raccoon feces decreased after 24 h, increased at 72 h, decreased at 120 h, and increased again after 168 h (Figure 12).

At 20°C, the highest cattle general *E. coli* growth was observed. Similar to other temperatures, the concentration enumerated increased through the 72 h record, decreased at 120 h and again increased after 168 h (Figure 11). At 20°C, *E. coli* from raccoons showed a similar trend as cattle with the only difference being that the highest counts for this temperature were observed at 168 h as opposed to 72 h in cattle (Figure 11 and 12).

The Kruskal-Wallis one way analysis of variance test was utilized to compare the differences between observed results. This non-parametric method is relevant and appropriate as it does not assume that data are normally distributed. Applying this test revealed no significant difference in cattle *E. coli* concentrations at 0° and 10°C ( $p > 0.05$ ) for any given incubation temperature over the 7 day study period. At 20°C, *E. coli* concentrations were significantly different for different days ( $p < 0.05$ ) (Figure 11). The same test found that there was a significant difference in *E. coli* concentrations in raccoon feces ( $p < 0.05$ ) among different days at all temperatures except at 0°C ( $p > 0.05$ ) (Figure 12). Since no survival was observed at 50°C after 24 h, the results obtained for that temperature were excluded from statistical analysis.

The Kruskal-Wallis test for *E. coli* concentration obtained after 1 h from both species did not support the hypothesis that the concentrations were different from each other at different incubation temperatures ( $p > 0.05$ ) (Figure 11 and 12). This result reinforced laboratory analysis as *E. coli* concentrations after 1 h were not expected to be different for different temperatures since they were background numbers. *E. coli* concentrations among temperatures were significantly different at all other analysis times. Results show that *E. coli* concentrations observed at 20°C were significantly higher at any time compared to the other incubation temperatures studied. For both the species studied, it was observed that at 50°C there was no survival of *E. coli* after 1 h.

In this study, *E. coli* growth was measured at different temperature conditions using fecal material directly added to water. If *E. coli* isolates from the feces were used instead, different growth results might have been observed as seen in our second study with isolates. This may be due to the fact that *E. coli* would not have to compete with other bacteria in fecal material. Also, the organic matter availability, as food for bacteria, would have been different under such conditions.

#### *Effects of Soil Moisture on Survival of E. coli*

The growth and survival of *E. coli* under different soil moisture conditions for cattle and raccoon species showed a similar trend to each other. The maximum survival and growth was observed at 25% moisture content of the soil sample followed by 56.5% moisture content. *E. coli* are facultative anaerobes which was reaffirmed from the results obtained that the bacteria had the highest growth and survival at 25% moisture content, indicating that among all soil moisture contents selected for this experiment, soil at 25% moisture content provided the most suitable conditions for their survival and growth.

Under dry conditions (4%), bacteria did not die-off completely, but by 168 h their concentrations reduced considerably. Reduction of two orders of magnitude for cattle samples (Figure 13) and by one half for raccoon (Figure 14) samples were observed. At 56.5% soil moisture content, *E. coli* concentration in cattle showed a gradual increase until 120 h followed by a reduction at 168 h whereas raccoon *E. coli* concentration declined at 120 h and increased at 168 h. At 83% moisture content, *E. coli* from cattle (Figure 13) reduced after a gradual growth until 120 h whereas the *E. coli* concentrations in raccoon samples (Figure 14) continued to rise from 1 h to 168 h.

Performing the Kruskal-Wallis test on *E. coli* numbers obtained in cattle feces at different moisture contents on different days yielded significantly ( $p < 0.05$ ) differences among all samples. The 1 h old samples showed the background concentration of *E. coli* for each moisture condition. A statistical difference in concentration of *E. coli* between different days indicates significant growth or decline. It can be observed that in cattle the 25% moisture condition had the highest *E. coli* concentration on any given day followed by 56.5%, 83%, and 4% moisture contents. The *E. coli* concentration from raccoons samples (Figure 14) showed a similar trend as cattle at 4%, 25% and 83% moisture content ( $p < 0.05$ ) but at 56.5% moisture condition there were no statistical differences in concentrations among different days.

The test statistics for growth and survival of *E. coli* from cattle and raccoons show that concentrations at all sampling times except 1 h ( $p > 0.05$ ) were different at each moisture content ( $p < 0.05$ ). Concentrations observed at 1 h were expected to be statistically similar at all moisture conditions since they represent background concentrations. Also, the difference in *E. coli* concentration at different moisture conditions between sampling times ( $p < 0.05$ ) suggests growth or decline of *E. coli* after 1 h.

The concentration of *E. coli* in this study did increase at 56.5% and 83% moisture but it was less than the concentration found at 25% at all sampling times. This study was conducted under room temperature conditions and the 25% soil moisture condition was likely to be the most favorable environment for bacteria to survive and grow at this temperature. Given the facultative anaerobic nature of these bacteria, it can be assumed that *E. coli* chose to be facultative at 25% soil moisture condition and room temperature as it provided optimum conditions for their survival and growth.

It should be noted that *E. coli* survival and growth in the environment can be influenced by the interacting effects of moisture conditions and temperatures. Other important physical and chemical properties such as pH and nutrient availability affect the survival of microorganisms should be taken into account to study the growth and survival of bacteria. In this study, under different environmental conditions, the temperature and moisture studies were independent of each other. In future studies, interaction of different temperatures and moisture conditions should be considered to study the effect of environment on survival and growth of bacteria. While modeling the fate and transport of *E. coli* in the environment, these complex effects should be considered.

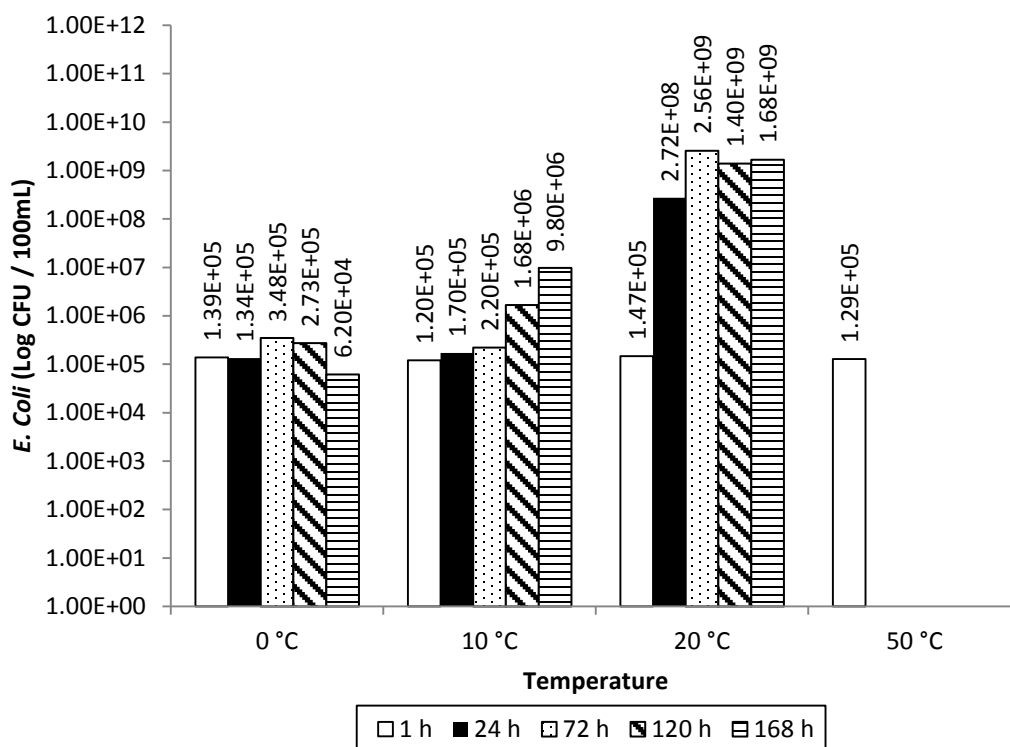


Figure 11. Survival of *E. coli* from cattle feces in water at different temperatures



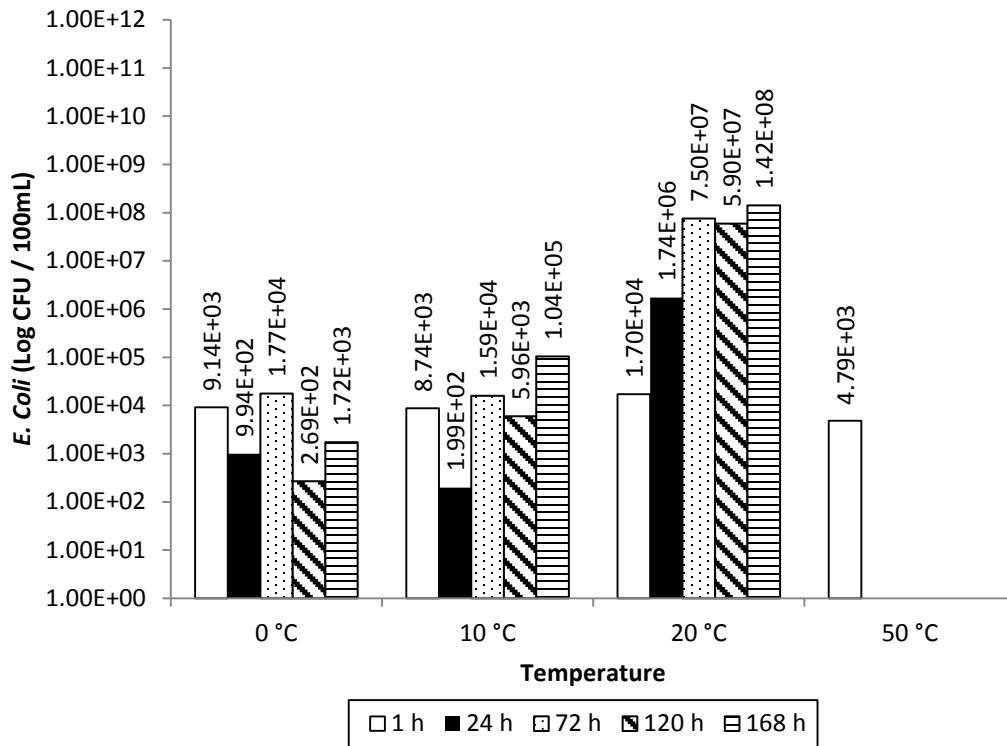


Figure 12. Survival of *E. coli* from raccoon feces in water at different temperatures

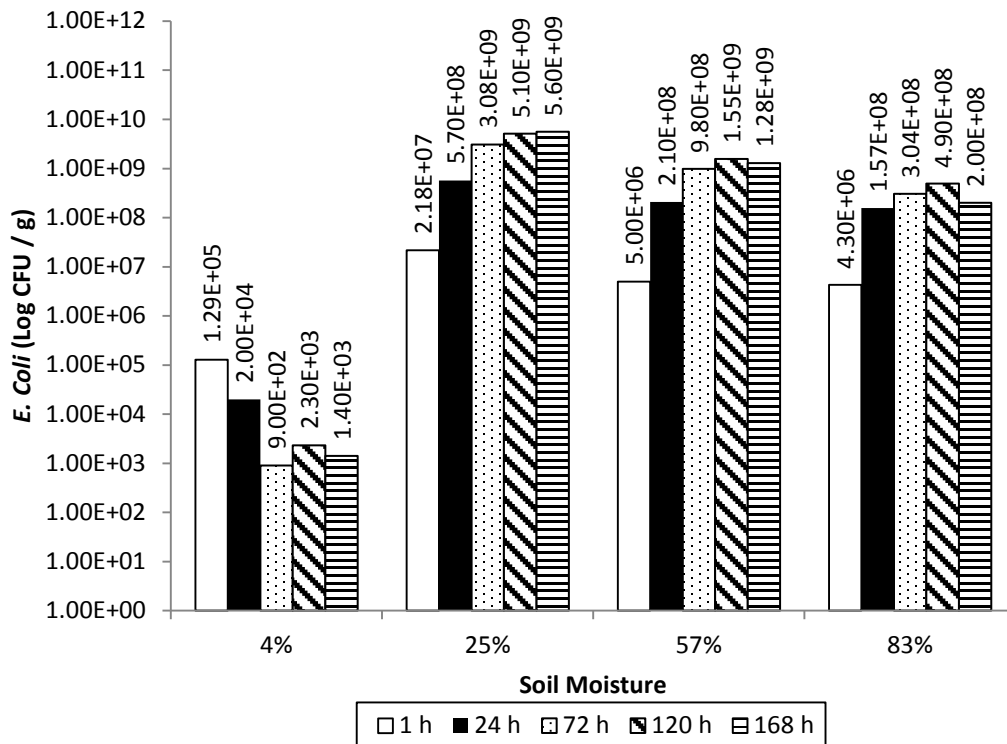


Figure 13. Survival of *E. coli* from cattle feces in soil at different moisture contents

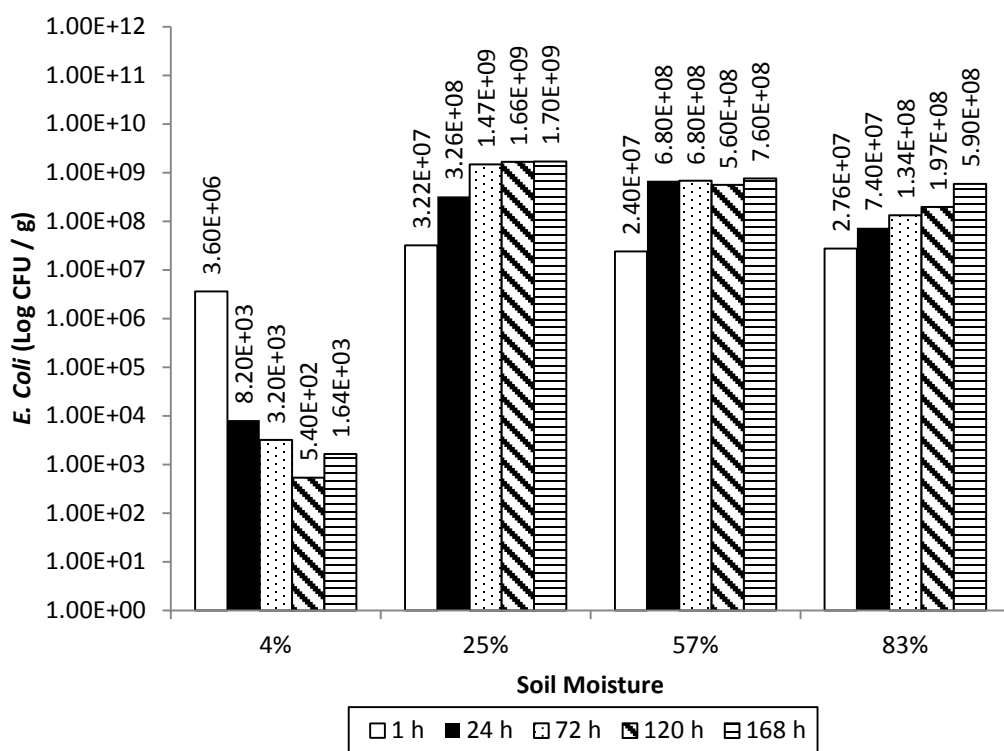


Figure 14. Survival of *E. coli* from raccoon feces in soil at different moisture contents

Table 10. First order rate constant for *E. coli* concentration in cattle, raccoons, feral hog, and deer at different temperatures in water

	$k_r$ (hr <sup>-1</sup> )			
	Cattle	Raccoon	Feral Hog	Deer
0 °C	-0.0025	-0.0073	*	*
10 °C	0.0151	0.0218	-0.0261	-0.0286
20 °C	0.0425	0.0472	*	*
25 °C	*	*	0.0812	0.0985
30 °C	*	*	0.109	0.198

\* Results produced from trials at these temperatures were invalidated due to fluctuations in temperature throughout the course of individual trials and across replicates

Table 11. First order rate constant for *E. coli* concentration in cattle, raccoons, feral hog, and deer at different moisture conditions in soil

	$k_{MC} \text{ (hr}^{-1}\text{)}$			
	Cattle	Raccoon	Feral Hog	Deer
4%	-0.0237	-0.0385	-0.2016	-0.2539
25%	0.0289	0.0207	-0.0065	-0.0187
56.50%	0.0281	0.0162	0.0177	-0.0326
83%	0.0182	0.0162	*	*

\* Results produced from trials at these temperatures were invalidated due to fluctuations in moisture conditions throughout the course of individual trials and across replicates

## Discussion

### Cattle and raccoon *E. coli* isolates in water and soil

The kinetic constants for cattle and raccoon *E. coli* at 20°C temperature (Table 10) and 25% moisture content were found to be quite similar to each other. It can be observed from kinetic constants that at 0°C temperature and at 4% moisture content there is decay in the *E. coli* population or simply a decline in *E. coli* numbers over time. At all other temperatures (except 50°C) and moisture contents growth can be observed. In this study, a greater rate of decay was observed for *E. coli* in soil at 4% moisture content compared to soil at 25% or 57% moisture content (Table 11). Based on the results from this study, *E. coli* concentration in dry soil with 4% moisture content decreased to half the initial concentration after approximately 3 – 4 h (Table 11). This rapid decay of *E. coli* in dry soil was observed to continue until the *E. coli* concentrations reached zero. For both cattle and raccoon fecal samples, maximum survival and growth of *E. coli* was observed at 20°C and no growth was seen at 50°C.

### Hog and deer *E. coli* isolates in water and soil

The kinetic constants for both feral hogs and deer were the lowest at 10°C and the highest at 30°C in water. These constants were negative in water at 10°C, indicating the k-value was a decay rate (Table 10). With the exception of the feral hog isolates at 56% moisture condition, both isolates experienced decay at different moisture conditions in soil.

From this study even though a conclusive optimum condition was not found, 30°C was used in water and 56% moisture in soil to grow both feral hog and deer isolates.

### Measure re-growth of *E. coli* in different sources under optimum conditions

Based on the results of the fate of *E. coli* in water and soil under different moisture conditions, optimum conditions were chosen based on the highest growth response observed. For cattle and raccoons, this was 20°C in water and 25% moisture in soil. *E. coli* isolated from feral hogs and deer responded differently and no conclusive optimum conditions were found. For the purpose of the re-growth evaluation, 30°C in water and 56% moisture in soil were chosen as these levels illustrated the most consistent *E. coli* response.

While these conditions were chosen as optimum, it should be noted that other temperature and moisture conditions may also lead to “optimum” or very near optimum survival response from *E. coli*. Evaluations conducted were based largely on “optimum” conditions reported in peer reviewed literature. As a result, many temperature and moisture levels were not assessed and may have yielded improved *E. coli* response.

## **Materials and Methods**

### ***E. coli* re-growth in water under optimum temperature**

*E. coli* isolates from hog and deer fecal samples were enriched in 100 mL of Luria-Bertani (LB) broth at 35.5°C for 24 h. The enriched LB broth was diluted to 10<sup>4</sup> by adding 1 mL of LB broth into 100 mL sterile DI water, stirred, then 1 mL will be added to 100 mL of sterile DI water. Creek water microcosms were made with 1 mL of the 10<sup>4</sup> dilution of LB broth in 100 mL of sterile Cedar Creek water (autoclaved three times at 121°C for 15 minutes). Microcosms were triplicated and kept in an incubator set at 30°C (optimum temperature established). Over 60 h, triplicate 0.1 mL samples of each water microcosm were spread plated onto MacConkey agar (Difco) plate at six different sampling times and the median value of *E. coli* counts (CFU/mL) were recorded at each time sampling.

*E. coli* isolates from fecal samples of cattle and raccoon were treated identically to those from feral hogs and deer with the exception of the incubation temperature. As determined in *E. coli* survival trials, the optimum temperature for these species was found to be 20°C; therefore, these samples were incubated as described above, but at 20 °C. Feral hog and deer isolates were incubated at 30°C.

### ***E. coli* re-growth in soil under optimum moisture condition**

*E. coli* isolates from hog and deer fecal samples were enriched in 100 mL of Luria-Bertani (LB) broth at 35.5°C for 24 h. A sterilized bottle was filled with 30 g of sterilized (autoclaved three times at 121°C for 15 minutes) soil from Cedar Creek and 1 mL of the inoculated broth was added to the soil in each bottle. Then 6 mL of sterile DI water was added to the soil with inoculum to obtain 56% moisture content at the optimum temperature established by evaluating *E. coli* response to environmental conditions. Soil samples were incubated at room temperature. *E. coli* in soil were enumerated after 1, 24, 72, 120, and 168 h. Enumerations were conducted in triplicate for each sampling time and the median *E. coli* numbers was reported as CFU per g dry weight of soil.

*E. coli* isolates from fecal samples of cattle and raccoon were treated identically with the exception of the moisture content. Results from the survival trials indicated that 25% moisture content was optimum to support survival and regrowth. As such, these samples were maintained at this moisture content.

## **Results**

Observations from these re-growth evaluations produced results that were quite different across the species and “optimum” conditions. Due to “optimum” condition variations across species, direct comparisons of re-growth potential cannot be made between *E. coli* from all species evaluated in water

and soil. One observation that can be made about all results is that the 1<sup>st</sup> order decay/growth rates observed are all relatively small with their absolute values ranging from 0.001 to 0.06 k(hr<sup>-1</sup>).

Table 12. First order rate constant for *E. coli* isolated from cattle, raccoons, feral hog, and deer at optimum temperatures in water during re-growth experiments

	$k_{MC} \text{ (hr}^{-1}\text{)}$			
	Cattle	Raccoon	Feral Hog	Deer
4%	-0.0237	-0.0385	-0.2016	-0.2539
25%	0.0289	0.0207	-0.0065	-0.0187
56.50%	0.0281	0.0162	0.0177	-0.0326
83%	0.0182	0.0162	*	*

\* \* Results produced from trials at these temperatures were invalidated due to fluctuations in moisture conditions throughout the course of individual trials and across replicates

Table 13. First order rate constant for *E. coli* concentration in cattle, raccoons, feral hog, and deer at different moisture conditions in soil during re-growth experiments

	$k_{MC} \text{ (hr}^{-1}\text{)}$			
	Cattle	Raccoon	Feral Hog	Deer
25%	0.032	-0.001	*	*
56%	*	*	-0.0025	-0.004

\* Results produced from trials at these temperatures were invalidated due to fluctuations in moisture conditions throughout the course of individual trials and across replicates

## Discussion

### ***Re-growth of E. coli isolates in water under “optimum” temperature conditions***

In general, the kinetic constant for all isolates are much lower during re-growth studies compared to that of growth studies (Table 10 and Table 12). In re-growth studies raccoon isolates exhibited decay instead of growth under optimum temperature found by evaluating *E. coli* response to environmental conditions. It should be noted that in growth studies, cattle and raccoon feces were directly dropped in water and the microcosms were incubated under different temperatures. In re-growth studies however the isolates from cattle and raccoon feces were used. That might be a reason why different *E. coli* growth kinetics were observed during growth and re-growth studies. At the same, growth and re-growth kinetics were different for hog and deer isolates even though the same isolates were used in both the studies. This clearly shows the ubiquitous nature of *E. coli* and inconsistency in its environmental fate.

### ***Re-growth of E. coli isolates in soil under “optimum” moisture conditions***

In general, the kinetic constant for all isolates are much lower during re-growth studies compared to that of growth studies (Table 11 and Table 13) except for *E. coli* isolated from cattle feces. In re-growth studies all isolates except from cattle exhibited decay instead of growth under optimum temperature found by evaluating *E. coli* response to environmental conditions. These studies again show the

inconsistency in *E. coli* growth in soil. In general the results point out that after initial optimum growth conditions cease, *E. coli* growth does not reach the maximum even when optimum conditions are established later. This may be due to *E. coli* losing its viability after certain time in the environment.

## **Monitoring concentration of *E. coli* in the instrumented stream as a result of rainfall and runoff events**

This effort was developed to evaluate the *E. coli* growth and survival response under different environmental conditions in streambed sediments. High *E. coli* numbers in stream are often associated with high turbidity water such as that seen during and following a storm flow event, or following a physical disturbance to the stream by human or animal activity in the creek. To accomplish this objective, in stream water samples were collected under base and storm flow conditions. Sediment samples from the stream bed were also collected immediately following each of these sampling events.

Work under this analysis was conducted by the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) on Resley Creek in Hamilton and Erath counties, Texas.

### ***Materials and Methods***

#### ***Water Sampling***

Water samples collected from the stream were gathered using grab and automated methods. Grab samples were collected under base flow conditions while automated sampling was utilized for storm flow samples.

To collect storm samples, automated sampling equipment was employed as described in Harmel et al. 2003. Briefly, this included installing ISCO 6712 automated samplers and programming them to collect samples when stream depth increased above 1.65 ft. This level was chosen based on prior knowledge of the stream and its base flow conditions. Once sampling commenced, it was continued until staff pulled the samples from the machine. Sampling was conducted to collect a flow-weighted composite sample into 8 – 2 liter sterile HDPE containers. When sampling began field staff were remotely notified by the sampling unit. Samples were retrieved and delivered to the lab with 22 h of initial collection to allow for processing within 24 h.

Grab samples were collected in triplicate by field staff using methods described in TCEQ's *Surface Water Quality Monitoring Procedures, Volume 1* (2003 and 2008). In short, this includes collecting samples directly into sterile Whirl-Pak sample containers from the mid-point of the stream at one-third of the total stream depth (i.e. Sample collected at 1 ft. in a 3 ft. deep stream). Staff collected sample prior to and following mechanical stream disturbances. Samples collected prior to disturbance were upstream from the location they were standing and took care to ensure that sediment they disturbed when entering the stream had moved downstream.

All samples were placed in a cooler on ice and kept a 4°C or less following labeling and during transport to the lab. Once in the lab, samples were processed and analyzed using USEPA Method 1603 for *E. coli* enumeration.



### ***Sediment Sampling***

Following each water sampling event, sediment samples were collected from 24 randomly selected locations along a 10 meter reach of the instrumented stream and composited into one sediment sample. The top 2 cm of sediment were collected using a sediment corer. The composited sample was placed in a sterile 1 liter container and transported to the lab for analysis. Sample containers were labeled and transported in a separate cooler on ice at 4°C.

### ***Mechanical Stream Disturbance***

Mechanical stream disturbance was accomplished by simply agitating the stream sediment with a garden rake. Field staff entered the stream directly upstream of the person collecting water samples influenced by mechanical disturbance. Staff began raking the stream bed perpendicular to flow and moved upstream away from the person collecting water samples. A 10 meter length of stream was disturbed while water samples were collected.

### ***Results***

The results of this study show that *E. coli* concentrations in storm samples were higher than in base flow samples. After sediment disturbance, the *E. coli* concentration in the water column significantly increased in all cases (Table 14). This clearly shows that stream bed sediments harbor *E. coli* for a long time. From this field study, it was determined that stream bed sediments represent a potential source of *E. coli* in stream water. *E. coli* concentrations in stream bed sediments were significantly higher than in water column under all conditions. These stream bed sediments have been overlooked while studying *E. coli* fate in the surface waters and should be thoroughly investigated.

Table 14. *E. coli* concentration in stream water before and after disturbance and in stream bed sediments

Sampling Date	7/28/2008	10/23/2009	1/18/2010	1/29/2010	2/5/2010	2/9/2010	3/9/2010	3/23/2010	3/26/2010
Event Type (Base or Storm)	B	S	B	S	S	B	S	S	S
<b>Water (CFU/100mL) Before Disturbance</b>									
Replicate 1	74	21	100	3300	150	20	NA	NA	NA
Replicate 2	0	14	0	3000	200	10	NA	NA	NA
Replicate 3	16	17	0	2850	190	17	NA	NA	NA
ISCO	NA	NA	NA	4350	195	NA	1300	370	NA
<b>Water (CFU/100mL) After Disturbance</b>									
Replicate 1	45	650	110	NA	270	81	2100	110	100
Replicate 2	47	1350	0	NA	200	39	2500	NA	30
Replicate 3	86	1310	0	NA	330	25	1900	NA	20
<b>Streambed (CFU/g dry weight)</b>									
Replicate 1	24	149000	8200	23000	7700	2000	11000	8000	3600
Replicate 2	10	240000	3800	14000	3200	440	10000	4500	1800
Replicate 3	21	360000	1200	16000	3400	100	7000	6700	2900

## Measure growth kinetics, survival, and re-growth of *E. coli* in stream bed sediments under different environmental conditions

Similar to efforts undertaken to quantify growth, survival and re-growth of *E. coli* in water and soil, these same kinetic factors were evaluated for *E. coli* in stream bed sediments.

### Materials and Methods

The materials and methods utilized in this evaluation were similar to those used in evaluating growth, survival and re-growth in soil and water. Individual sediment samples were exposed to temperatures of 0°C, 10°C, 20°C and 30°C and held constant at those temperatures for 168 h. Samples were collected from each sediment sample at 1, 24, 72, 120 and 168 h. Using the Byappanahalli et al. (2003) to extract *E. coli* from feces, *E. coli* were extracted from these sediment samples and then enumerated using the USEPA 1603 membrane filtration method (USEPA, 2006).

### Results

In general, *E. coli* growth in stream bed sediments was higher as the temperature increased. In tropical and subtropical conditions stream bed sediments will experience higher temperatures, particularly during summer months. *E. coli*, if deposited in the stream bed, will grow under such conditions. During re-growth studies, *E. coli* growth varied when optimum conditions were re-established. The variability in growth and re-growth could be attributed to nutrient availability, predators, *E. coli* population, and other environmental conditions.

Table 15. First order rate constant for *E. coli* concentration in stream bed sediments at different temperatures

	$k_T$ (hr <sup>-1</sup> )			
	Rep 1	Rep 2	Rep 3	Rep 4
<b>0°C</b>	-0.0017	-0.0023	-0.01	0.003
<b>10°C</b>	0.021	-0.025	-0.031	-0.019
<b>25°C</b>	0.034	0.041	0.036	0.064
<b>35°C</b>	0.028	0.015	0.076	0.085

Table 16. First order rate constant for *E. coli* concentration in stream bed sediments at different temperatures during re-growth experiments

	$k_T$ (hr <sup>-1</sup> )		
	Rep 1	Rep 2	Rep 3
<b>25°C</b>	0.052	0.037	0.04

35°C	-0.013	0.025	0.037
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### Discussion

This evaluation was dissimilar to other evaluations conducted in that sediment samples taken directly from the stream were incubated at varying temperatures and *E. coli*'s response to these changing temperatures was quantified. In all other evaluations, a sterile environment (autoclaved water or soil) was used as a growth medium. In this case, sediment as removed from the stream provided the growth medium. As a result, *E. coli* was subjected to direct competition for resources from other soil biota as well as possible predation.

#### **Growth and survival of *E. coli* isolates in stream bed sediments**

*E. coli* did not show any growth in three of the microcosms each kept at 0°C and 10°C (Table 15). *E. coli* survived and exhibited net growth in stream bed sediments at 25°C and 35°C. This suggests that at cooler temperatures, *E. coli* are not as adept at survival in the environment. However; as is commonly the case with bacteria, the general tendency of *E. coli* to “die-off” under these lower temperatures was not ubiquitous. One replication did exhibit slight growth thus exposing the inherent variability of *E. coli*'s behavior.

The trend of considerable variability was somewhat broken at 25°C and 35°C. All replications exhibited growth within an order of magnitude (.015 - .085 k(hr<sup>-1</sup>)) thus illustrating the propensity of *E. coli* to not only persist but thrive in elevated temperatures.

#### **Re-growth of *E. coli* in stream bed under “optimum” temperature conditions**

Based on results from the growth and survival of *E. coli* in stream sediment, 25°C and 35°C were used to evaluate *E. coli* re-growth under “optimum” temperature conditions. In general, the kinetic constant for *E. coli* growth showed similar trends during re-growth studies compared to that of growth studies (Table 15 and Table 16). In re-growth studies, *E. coli* decayed at 35°C in one stream bed microcosm while all other microcosms exhibited growth (Table 16). These results again reaffirm the inherent variability of *E. coli* response to changing environmental conditions.

## Summary and Future Recommendations

*E. coli* concentrations in feces of different animals evaluated varied widely within and between species. Seasonality (summer or winter) also played a significant role in the variability as well. The high variability of *E. coli* in the feces of same species could be attributed to diet, region, sampling time, sex, gender, habitat, and management practices. Accurate estimation of *E. coli* concentration will improve the spatial and temporal *E. coli* load estimations using models. The results from this study emphasize that *E. coli* load estimates to waterbodies in the watershed will be more representative with direct source characterization and identification of sources but will still contain an unavoidable level of uncertainty simply due to the inter and intra-species variability observed.

Kinetic constants obtained for *E. coli* isolates from hog, deer, cattle, and raccoon show significant variability. In general, under very cold conditions (0°C) in water and under very dry conditions (4% moisture) in soil, *E. coli* did not survive. The optimum conditions for *E. coli* growth and re-growth varied from species to species. Overall, the results point out that *E. coli* can persist in aquatic systems even after excreted from animals. As designed, this study evaluated temperature and moisture conditions independently. Future work should explore the interaction of different temperatures and moisture conditions simultaneously to assess the effect of the environment on survival and growth of *E. coli*.

Disturbance of stream bed sediments significantly increased the *E. coli* concentrations in water samples collected after the disturbance. This clearly indicates that re-suspension of *E. coli* from sediments could be a significant source of *E. coli* in streams. It should also be noted that stream bed sediments had significantly higher *E. coli* concentrations compared to water samples. Clearly, stream sediments are a source of *E. coli* during stream bed disturbance. Further work is needed to evaluate the dynamics of *E. coli* populations in stream sediment.

*E. coli* in stream beds showed growth at 25°C and 35°C but not at 0°C and 10°C. These results indicate that stream sediments could support increasing quantities of *E. coli* during summer months that could be a considerable source of *E. coli* concentrations in water samples. Despite the decay observed at lower temperatures, this does not preclude the potential for sediment to contribute *E. coli* to the water column under lower temperature conditions. Further research is needed to investigate the interplay of in stream water conditions with sediment borne *E. coli* populations.

The data presented from this research is a beginning of cataloging *E. coli* concentration in wildlife and range animal fecal material. In this study, fecal sample sizes for feral hog, deer, skunk, and armadillo were much lower than raccoon and opossum. Additional fecal samples should be collected and analyzed to capture the variability in *E. coli* concentrations for those species. Further research should be conducted to study the seasonal variability in *E. coli* concentration in fecal material of meso-mammal populations.

Modeling the fate of *E. coli* in the environment is a complex process. There are many environmental controls that can affect the fate of *E. coli* but only two were considered in this study. Other

environmental controls such as amount of carbon and other nutrients and soil type should be included while studying the fate of *E. coli*. This study focused on differences in the kinetic characteristics of *E. coli* isolates from cattle, raccoon, feral hog, and deer fecal material. Differences in kinetic characteristics for *E. coli* isolates from other potential sources' fecal material, such as other wildlife species and birds should also be studied. Additionally, this study was conducted under controlled laboratory conditions. Repetitive studies of the growth rate of *E. coli* in soil and water will verify the reproducibility of the results with higher success. Further studies should be built upon the understanding of the fate of *E. coli* under controlled conditions, incorporating more variables to better represent field conditions.

From this controlled-demonstration study we can conclude that studying *E. coli* fate and persistence in the terrestrial environment is a challenge and conflicting results are paramount. This study points out the need for future studies on fate of *E. coli* in soil and water. Effects of nutrients, temperature, and soil moisture conditions and their interacting effects should be thoroughly studied. Controlled studies with the inclusion of stream bed sediments are inevitable. Artificial streams (channel models) with flowing water of varying water chemistry and different stream bed sediments should be used to study the fate and transport of *E. coli* instead of small microcosms used in this study to simulate natural conditions. Lessons learned and challenges faced in this study will help carry out the future *E. coli* fate studies better. The wealth of information learned from this study will help the stakeholders understand the ubiquitous nature of *E. coli* in the environment.

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## APPENDIX A:

### **“Occurrence and Fate of *E. coli* from Various Non-point Sources in a Subtropical Watershed”**



## Occurrence and Fate of *E. coli* from Various Non-point Sources in a Subtropical Watershed

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### Abstract

Bacteria of fecal origin are the primary cause of surface water contamination in the US. *E. coli* is used as an indicator of fecal contamination and detection of *E. coli* in a water body above regulatory standards poses a potential health hazard. Various sources contribute to the bacterial contamination of a water body, and these sources need to be identified and quantified to estimate bacteria loads in the waterbody accurately. In-situ re-growth is also believed to be a considerable source of *E. coli* in many cases. Also re-growth of *E. coli* in landscapes due to favorable environmental conditions (e.g., rainfall after dry weather conditions) is one of the major phenomena affecting *E. coli* concentration in streams. The objective of this study was to identify, characterize, and quantify *E. coli* concentration from feces of four different animal species, and monitor survival, growth and re-growth at four different temperatures and moisture contents over a period of seven days. Wildlife and range cattle fecal samples from the Cedar Creek watershed in East Central TX, USA were identified and feces from four species out of those were quantified for the *E. coli* concentrations. No significant difference was found while comparing the *E. coli* concentration for each species between the genders. Sub-adult cattle feces had significantly higher *E. coli* concentrations than those from adult cattle. Growth and die-off rates of *E. coli* were measured at different temperatures (0°C, 10°C, 25°C, and 50°C) in creek water and moisture conditions (4%, 25%, 56.5% and 83%; volumetric basis) in soil. *E. coli* concentrations in cattle and raccoon feces showed the highest survivability and growth at 20°C. There was no survival of *E. coli* from either species at 50°C after 24 h. *E. coli* in cattle and raccoon fecal samples exhibited greater growth at lower, nearly aerobic soil moisture content (25%) for all days compared to nearly anaerobic soil moisture content (83%).

**Key words:** Fecal bacteria, Water quality

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### INTRODUCTION

Bacteria are the leading cause of impairment of surface waters, including rivers, lakes, and streams in the US (USEPA 2008). As of January 2007, 197 water bodies in the State of Texas were impaired because they did not meet the bacteria concentration criteria established by the state to protect contact recreation use. A geometric mean of 126 colony-forming units (CFU)/100 mL and a single maximum of 394 CFU/100 mL for *Escherichia coli* (*E. coli*) are the criteria used to determine the impairment for freshwater

contact recreation use (TSSWCB 2007). Fecal contamination of a waterbody is commonly determined by detecting the presence of indicator organisms. Fecal contamination is the pollution caused due to microorganisms like bacteria, protozoa, virus and fungi present in the intestine of humans and animals. *E. coli* is used as an indicator organism to identify fecal contamination of water bodies (Byappanahalli et al. 2003; Chin et al. 2009). Presence of indicator organisms suggests potential occurrence of pathogenic strains of the

bacteria, protozoa, virus, and fungi (Bolster et al. 2009).

To meet the criteria set by the regulatory agencies, watershed models are often applied to study the current status of water quality and the impacts of various management plans. Watershed models such as Soil Water Assessment Tool (SWAT) and Hydrologic Simulation Program in Fortran (HSPF) or the load-duration curve method are typically used in Total Maximum Daily Load (TMDL) and Watershed Protection Plan (WPP) development (Benham et al. 2007). Most of the models are limited in their ability to simulate bacteria concentrations during varying environmental conditions. These models use literature values for the concentration of *E. coli* in various fecal sources. It is necessary to accurately identify and characterize the sources and also quantify them to accurately predict the bacterial loads using watershed models. Studying the survival and growth of *E. coli* under variable environmental conditions will help in modeling their fate and transport processes more accurately (Riebschleager 2008).

The growth of *E. coli* in the environment is not completely understood or documented (Ishii et al. 2006). It has become progressively clearer that given the right conditions, such as availability of nutrients, temperature, moisture, etc., these bacteria can survive and possibly replicate in soil and water (Byappanahalli et al. 2003; Ishii et al. 2006; Sherer et al. 1992; Stephenson and Rychert 1982; Wang et al. 2004). The fate and transport of *E. coli* has been investigated by several studies (Bolster et al. 2009; Habteeselassie et al. 2007; Ishii et al. 2006; Sherer et al. 1992;

Wang et al. 1996) but still better understanding is required to improve the modeling of fate and transport processes.

In this study *E. coli* concentrations of various fecal sources were determined. We also examined the survival and growth of *E. coli* at four different temperatures in water and at four different moisture contents in soil at a constant temperature. The water temperatures selected for this study were 0°C, 10°C, 20°C, and 50°C to represent the actual seasonal temperatures found in the study area and waste treatment practices such as composting. The four soil moisture contents selected were 4%, 25%, 56.5%, and 83% (volumetric basis) with the purpose of studying growth and survival under dry, damp, wet and saturated soil environment.

## MATERIAL AND METHODS

### Study Area Description

Cedar Creek Watershed, located in Brazos County and Robertson County in East Central Texas, has a total area of 340.54 km<sup>2</sup>, of which about 95.3% is undeveloped forest land, 3.9% developed area and 0.82% open waters (Figure 1). The local climate is subtropical and temperate. Summers are warm and hot with occasional showers. Winters are mild with periods of low temperatures usually lasting less than two months. The annual rainfall in this area is from 810 to 1220 mm. The dominant soil type is clayey loam soil.

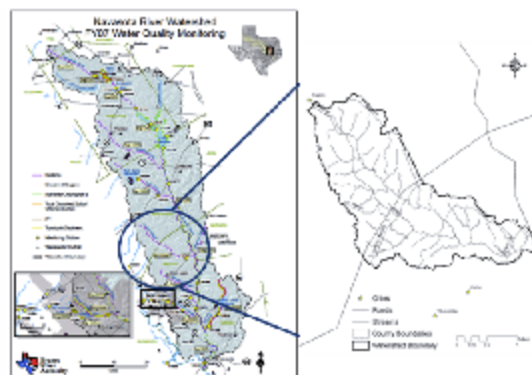


Figure 1 Study Area: Cedar Creek Watershed, Texas, USA

Cedar Creek is one of the several water bodies deemed impaired because it does not meet bacteria criteria (TSSWCB 2007; TCEQ 2008). It falls under 5c category, which means additional data and information needs to be collected before a

TMDL is scheduled by the Texas Commission on Environmental Quality (TCEQ 2008). Cedar Creek has little or no urban influence. Except for direct deposition from animals there is no other evidence of point source

contamination into this Creek. Bacterial contamination is mainly resulting from agricultural and rural sources such as cattle and wildlife.

#### Sampling Protocol

Two sub-watersheds were selected for the study based on the watershed survey and co-operation of land owners. These sub-watersheds were located on the southwest of Cedar Creek watershed. Various non-point sources of *E. coli* (wildlife and cattle) were identified in the study area. Out of those sources, fecal samples were collected specifically from Armadillos, Opossums, Raccoons, and Cattle. The fecal material was collected by trapping the wild animals from the two sub-watersheds during summer for three months. Trapping of animals and collection of fecal material described below was conducted according to a standard protocol established by a wildlife expert (Lopez 2008). During the same sampling period, fecal material was collected from grazing cows in the watershed right after deposition.

A grid-design was used for 42 traps per sub-watershed, each measuring 81 cm × 25 cm × 30 cm. (raccoons/feral cat Tomahawk Live Trap, Tomahawk, WI). The traps were spaced at 150 m. This spacing distance has shown adequate sampling of animals that are highly attracted to aromatic baits (e.g., raccoons and opossums). Randomly located trap arrays were used in order to capture armadillos, rabbits, and skunks (i.e., species less attracted to bait). Variable array setups were designed to take advantage of the local vegetative community and topography. The arrays were fabricated out of 61 cm tall chicken fencing with 61 cm long wooden stakes. Each array had 8-12 armadillo/rabbit traps (43 traps total for each sub-watershed; 48 cm × 15 cm × 15 cm; Tomahawk Live Trap, Tomahawk, WI).

The traps were laid in the evening and kept there till next morning. The trapped animals were released next day early morning and fecal material was collected in Whirl-Pak Bags® (Nasco, WI). Date of trapping, species information, trap number, tag number (in case of cattle), age and gender of the animal were labeled on each sampling bag. The Whirl-Pak Bags® were kept in coolers with ice and transported to the laboratory.

#### Enumerating *E. coli* from Fecal Samples

All fecal samples were brought to the laboratory, kept frozen until analyzed and enumerated for *E. coli* using a method used by Byappanahalli et al. (2003). All the samples were analyzed between 24 and 72 h after they were brought to the laboratory. Fecal samples were first thawed to room temperature and then a 1 g sub-sample was taken from each fecal sample and added to 9.5 mL of sterile de-ionized water in a test tube. The test tube with its contents was vortexed for two minutes to elutriate bacteria from the fecal sub-sample.

The suspension was serially diluted and filtered using Millipore® 0.45 µm membrane filters. A standard membrane-filtration method (EPA Method 1603) to enumerate *E. coli* in water was used to estimate *E. coli* concentrations. Briefly, vortexed aqueous solution was filtered through a membrane filter placed on a filter base using sterilized forcep to retain the bacteria and then direct count of *E. coli* was obtained based on the development of colonies that grew on the surface of the membrane filter placed on a selective nutrient medium (USEPA, 2002).

The nutrient medium for analyses was prepared by adding 45.6 g of dehydrated modified membrane-Thermotolerant *Escherichia coli* (modified mTEC) agar powder (Becton-Dickinson, NJ) to 1 L of de-ionized water and then boiling the mixture for one minute. Modified mTEC agar is a selective and differential medium used for chromogenic detection of *E. coli*. The agar was autoclaved at 121°C for 15 minutes, poured into 9 × 50 mm Petri plates, and allowed to solidify at room temperature. Petri plates with membranes were incubated in inverted position for 2 h at 35 ± 0.5°C to resuscitate the stressed cells. After two hours of incubation, Petri plates were transferred into a Whirl-Pak® bag. The bag was sealed and incubated in a water bath at 44.5 ± 0.2°C for 22 to 24 h. The Petri plates were removed from the water bath and the number of red/magenta colonies developed on the membrane were counted and recorded. Aseptic techniques were followed throughout experiments and if any growth observed on a control plate then that counting was rejected. Only the plates having colonies between 30 and 300 were used to report *E. coli* concentrations as CFUs per g of wet fecal material. The gravimetric moisture content of all fecal samples were determined simultaneously by drying 1 g of the wet sample at 100°C for 24 h. Moisture content was calculated as (Wet weight of fecal sample – Dry weight of fecal sample) × 100 ÷ Wet weight of fecal sample.

Once colonies were obtained on mTEC agar, one randomly selected colony from each sample was isolated by streaking on Luria-Bertani (LB) Agar (Becton-Dickinson, NJ) and incubated at 35°C for 24 h. After the colonies were obtained on LB Agar, one randomly selected colony was again streaked on MacConkey agar (Becton-Dickinson, NJ) to confirm the presence of *E. coli* in the samples. If colonies were obtained on both media then it positively confirmed that the bacteria isolated were *E. coli*.

#### Fate of *E. coli* under Different Environmental Conditions

Three fecal samples were randomly selected from each of the two species: cattle and raccoons. Each sample was exposed to different temperatures and moisture conditions. The experiments for testing the growth and survival of *E. coli* under different temperature conditions were done by mixing

the fecal samples with sterilized water collected from Cedar Creek. To study the effect of moisture conditions, isolates of *E. coli* of the same samples were inoculated with soil from Cedar Creek watershed. Glassware and supplies used in the experiments were sterilized by autoclaving at 121°C for 15 minutes.

#### *E. coli* Survival in Water at Different Temperatures

To mimic direct fecal deposition in streams and to study the effects of temperature on *E. coli* survival in the streams, ten grams of fecal sample was mixed with 95 mL of sterilized (autoclaved three times at 121°C for 15 minutes) Cedar Creek water. The mixture was then divided into four equal volumes in sterilized bottles and stored at 0°C, 10°C, 25°C and 50°C. *E. coli* in water was enumerated after 1, 24, 72, 120, and 168 h using EPA Method 1603. The enumeration for each time sampling was in triplicates and median *E. coli* concentrations were reported as CFU per 100 mL.

#### *E. coli* Survival in Soil at Different Moisture Conditions

Isolates of the same samples used to study *E. coli* survival in water at different temperatures were used to study the survival of *E. coli* at different moisture contents in soil. This would simulate *E. coli* fate within the soil matrix after fecal matter is deposited on soil surface and *E. coli* survival after isolated from feces at varying soil water contents. *E. coli* isolates were streaked on LB agar and allowed to grow for 24 h at 35°C. Out of the colonies obtained after 24 h one randomly selected colony was cultured in LB broth at 35°C for 24 h (Bolster et al. 2009). A sterilized bottle was filled with 30 g of sterilized (autoclaved three times at 121°C for 15 minutes) soil from Cedar Creek and 1 mL of the inoculated broth was added to the soil in each bottle. Then, 0, 6, 15, and 22.5 mL of sterile de-ionized water was added to the soil with inoculum to obtain 4%, 25%, 56.5% and 83% moisture content (on volumetric basis; dry bulk density of soil was 1.06 g/mL), respectively. Soil samples were incubated at room temperature. *E. coli* in soil was enumerated after 1, 24, 72, 120, and 168 h. The soil samples were enumerated for *E. coli* the same way as the fecal materials were enumerated. The enumeration for each time sampling was in triplicates and the median *E. coli* concentrations were reported as CFU per g wet weight of soil. First order rate constants for *E. coli* survival in water at different temperatures and in soil at different moisture conditions were determined by calculating the slope of the linear regression line of log *E. coli* concentration (y axis) vs. time (x axis) plot.

#### Statistical Analysis

Results from the experimental study were analyzed using SPSS Statistics 17.0 software (SPSS Inc. 2008). Based on

preliminary statistical analysis, *E. coli* concentrations of fecal samples resulting from all species were not normally distributed. So a non-parametric test was performed to analyze *E. coli* concentrations. Kruskal-Wallis test was used to find if there was any significant difference in *E. coli* concentrations resulting from the four species (McDonald 2009). To find whether there was difference in a particular species either based on gender or age Mann-Whitney test was performed. Mann-Whitney test was used only when two variables were compared. It is the non-parametric equivalent to Student's t-test (McDonald 2009).

During the *E. coli* survival and growth experiments the temperature and moisture treatments were exclusive of each other, i.e., the moisture conditions were not changed while measuring the survival and growth at different temperatures and the temperature was not changed while studying the survival and growth at different moisture conditions. For the survival and growth of *E. coli* under different temperature and moisture conditions, *E. coli* concentrations were analyzed using SPSS Statistics 17.0 software (SPSS Inc., Chicago). Upon checking the normality of the *E. coli* concentrations obtained for both the treatments (temperature and moisture conditions) it was found that the data was skewed. Therefore to find whether there was a difference in *E. coli* concentrations on different days and within treatments the Kruskal-Wallis test was performed. The research hypotheses that were statistically tested were: (1) *E. coli* concentration from feces of a species subjected to different temperatures on any particular day will be different for different temperatures in water, (2) *E. coli* concentration from feces of a species subjected to different moisture conditions on any particular day will be different for different moisture conditions in soil, (3) *E. coli* concentration from feces of a species at a particular temperature in water will be different on different days, and (4) *E. coli* concentration from feces of a species at a particular moisture condition in soil will be different on different days.

## RESULTS AND DISCUSSION

#### *E. coli* Concentration in Feces of Different Species

The *E. coli* concentrations from cattle and wildlife feces samples collected from the Cedar Creek watershed were reported in CFU/g of wet fecal material and then converted in CFU/g of dry fecal material based on corresponding moisture content of the feces. Various samples from four different species were used to analyze for their *E. coli* concentrations. Table 1 presents the fecal *E. coli* concentration of different species collected during summer. All samples were analyzed under similar temperature conditions and collected independent of each other.



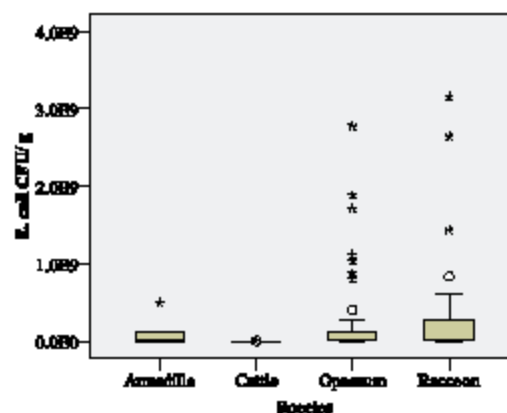
**Table 1** *E. coli* concentration in feces of different species

Species	Number of samples		CFU/ g of dry fecal material	
	Collected	Analyzed	Median	Range
Armadillo	7	5	$1.09 \times 10^7$	$4.32 \times 10^5 - 6.83 \times 10^8$
Raccoons	86	43	$2.29 \times 10^7$	$3.06 \times 10^5 - 5.46 \times 10^9$
Opossum	76	57	$3.71 \times 10^7$	$3.31 \times 10^4 - 3.24 \times 10^9$
Cattle	26	17	$1.61 \times 10^5$	$3.35 \times 10^2 - 1.74 \times 10^7$

The four species exhibited a lot of variability in the concentration of *E. coli* in their feces. Out of the four species analyzed, median *E. coli* concentrations from opossum ( $3.71 \times 10^7$  CFU/g) and raccoons ( $2.29 \times 10^7$  CFU/g) feces were higher than cattle ( $1.61 \times 10^5$  CFU/g) and armadillo ( $1.09 \times 10^7$  CFU/g). The *E. coli* concentrations from cattle feces were found to be the lowest of all the species analyzed.

Figure 2 shows the distribution of *E. coli* in four different species. It was observed that data for all the species were highly skewed with a number of outliers shown as asterisks and dots above the box plots. A non-parametric analysis of all *E. coli* concentrations of all four species showed a significant difference among the *E. coli* concentrations of the four species ( $p < 0.05$ ). *E. coli* concentrations from feces of different animals were different possibly due to their feed types. The omnivorous nature of armadillo, opossum, and raccoons could be attributed to higher *E. coli* counts than herbivorous cattle.

Additionally, data showed that median *E. coli* concentrations in the feces of wildlife and cattle varied with age and gender (Table 2).



**Figure 2** *E. coli* concentration in feces of different species

**Table 2** *E. coli* concentration in feces resulting from species of different age and gender.

Species		CFU/g of dry fecal material			
		Male	Female	Adult	Sub-adult
Opossum	n	32	25	47	10
	Range	$1.41 \times 10^5 - 3.13 \times 10^9$	$3.31 \times 10^4 - 3.24 \times 10^9$	$3.31 \times 10^4 - 3.24 \times 10^9$	$3.23 \times 10^5 - 2.87 \times 10^9$
	Median	$4.91 \times 10^5$	$4.29 \times 10^7$	$2.71 \times 10^7$	$2.37 \times 10^8$
Raccoons	n	29	14	40	3
	Range	$3.06 \times 10^5 - 5.46 \times 10^9$	$6.95 \times 10^5 - 3.21 \times 10^9$	$6.46 \times 10^5 - 5.46 \times 10^9$	$3.06 \times 10^5 - 4.51 \times 10^7$
	Median	$1.93 \times 10^7$	$1.92 \times 10^7$	$2.54 \times 10^7$	$8.68 \times 10^5$
Armadillos	n	4	1	5	0
	Range	$4.32 \times 10^7 - 1.52 \times 10^8$	NA	$4.32 \times 10^7 - 6.83 \times 10^8$	NA
	Median	$5.91 \times 10^6$	$6.83 \times 10^8$ (*)	$1.09 \times 10^7$	NA
Cattle	n	5	12	13	4
	Range	$2.67 \times 10^4 - 9.18 \times 10^5$	$3.35 \times 10^2 - 1.74 \times 10^7$	$3.35 \times 10^2 - 1.74 \times 10^7$	$1.61 \times 10^5 - 9.18 \times 10^5$
	Median	$1.61 \times 10^5$	$1.03 \times 10^5$	$1.82 \times 10^5$ (a)	$5.40 \times 10^4$ (b)

\* note that this is only one value and not really a median.  
(a) & (b) indicates statistically significant values at ( $p < 0.05$ )



As shown in Table 2, sub-adult opossums shed more bacteria than adults, but the difference was not statistically significant ( $p > 0.05$ ). Also, there was no statistical difference between the *E. coli* concentrations in the feces of male and female opossums (Table 2). Adults and female raccoons demonstrated higher median *E. coli* concentration than their male and sub-adult counterparts, respectively, but the difference was not statistically significant ( $p > 0.05$ ) (Table 2). Data for only adult animals were available for armadillos. While a difference in the *E. coli* concentration from males and females can be observed in the box plots (graphs not shown here), it was not statistically significant according to the Mann-Whitney test ( $p > 0.05$ ) (Table 2).

Feces from calves had a significantly higher ( $p < 0.05$ ) *E. coli* concentration than adult cows (Table 2). Even though it seemed from the median values that *E. coli* concentration from male cattle was higher than females, the difference was not statistically significant ( $p > 0.05$ ) (Figure 4b). To our knowledge, this is the first study which examined the variation of *E. coli* count with respect to age and gender of animals, particularly wild animals.

Of all the species studied, only cattle showed a statistically significant difference in the *E. coli* concentration between adults and sub-adults. There are several studies which have examined cattle gut microflora. Cary and Moon (1995), Wells et al. (1991) and Zhao et al. (1995) had observed that *E. coli* O157:H7 concentration was significantly higher in feces of calves compared to adult feces. Rasmussen et al. (1993) and Fenlon and Wilson (2000) explained that adult cattle have a fully developed rumen where the combination of a highly volatile fatty acid concentration and a low pH inhibits the growth of *E. coli* O157:H7. No statistically significant difference in *E. coli* concentration was observed between genders of all four species studied. This may be mainly because there is no reported difference in digestion patterns or enteric bacteria occurrence between males and females of the same species.

#### Fate of *E. coli* under Different Environmental Conditions

The results of analysis of *E. coli* concentration from cattle and raccoons at different temperature and moisture conditions over a period of seven days showed different trends and variability. Median *E. coli* concentrations in CFU per 100 mL of water or g of dry soil were plotted in Figures 3 through 6.

##### Effects of Temperature on *E. coli* Survival in Water

The survival of *E. coli* from cattle and raccoon in water at different temperatures over a period of seven days showed highly variable *E. coli* counts (Figures 3 and 4). *E. coli* concentrations in cattle and raccoon fecal samples at the beginning of the experiments were determined after one hour.

These background concentrations are presented for comparison with the bacterial concentrations from subsequent days. For both species maximum survival and growth of *E. coli* was observed at 20°C and no growth was seen at 50°C.

At 0°C, there was a slight decrease in cattle *E. coli* growth after 24 h. The concentration increased after 72 h and then decreased until the end of the incubation period (Figure 3). *E. coli* from raccoon feces at 0°C (Figure 4) showed a decrease after 24 h, increased at 72 h, decreased at 120 h, and increased again after 168 h.

A gradual increase in the cattle *E. coli* concentration was observed at 10°C until the fifth day (120 h) and then there was a decline by one order of magnitude after 168 h (Figure 3). While the *E. coli* from raccoon feces decreased after 24 h, increased at 72 h, decreased at 120 h, and increased again after 168 h. This survival trend of *E. coli* from raccoon feces was similar to that of 0°C (Figure 4).

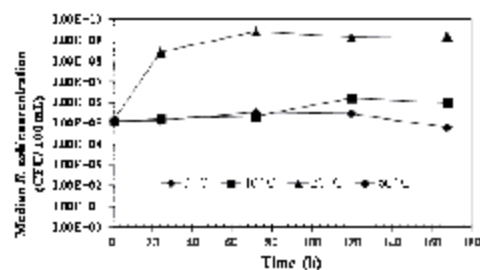


Figure 3 Survival of *E. coli* (in cattle feces) in water at different temperatures.

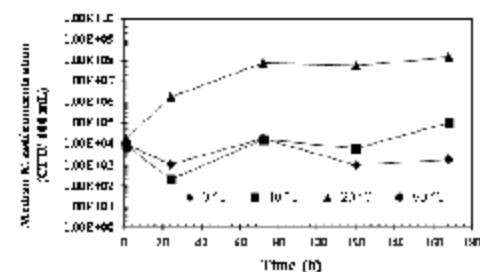


Figure 4 Survival of *E. coli* (in raccoon feces) in water at different temperatures.

The highest cattle *E. coli* growth was observed at 72 h at 20°C. The concentration dropped at 120 h and again increased after 168 h (Figure 3). At 20°C, *E. coli* from raccoons showed a similar trend as cattle with the only difference being that the highest counts for this temperature were observed at 168 h as opposed to 72 h in cattle (Figure 3 and 4). The decline on any given day might be due to the depletion of nutrients over time and increased competition for nutrients within bacterial population. The re-growth could possibly have occurred due to the nutrition available from the organic matter of the dead bacterial cells.

There was no significant difference in cattle *E. coli* concentrations at 0° and 10°C ( $p > 0.05$ ) for any given incubation temperature over the 7-day study period. However, at 20°C the *E. coli* concentrations were significantly different for different days ( $p < 0.05$ ) (Figure 3). The Kruskal-Wallis test statistics for *E. coli* in raccoon feces showed that there was a significant difference ( $p < 0.05$ ) in *E. coli* concentrations among different days at all temperatures except at 0°C ( $p > 0.05$ ) (Figure 4). Since no survival was observed at 50°C after 24 h, the results obtained for that temperature were excluded from statistical analysis.

Kruskal-Wallis test for *E. coli* concentration obtained after 1 h from both species did not support the hypothesis that the concentrations were different from each other at different incubation temperatures ( $p > 0.05$ ) (Figure 3 and 4). This result just reinforced the laboratory analysis as *E. coli* concentrations after 1 h were not expected to be different for different temperatures since they were background numbers. *E. coli* concentrations among temperatures were significantly different at all other days. The results show that *E. coli* concentrations observed at 20°C were significantly higher on any day compared to the other incubation temperatures studied. For both the species studied, it was observed that at 50°C there was no survival of *E. coli* after 1 h.

Habteselassie et al. (2007) found that *E. coli* survived better at lower temperatures in soil, whereas in our study the survival of *E. coli* resulting from cattle and raccoon was the highest at 20°C compared to the survival at 0°C and 10°C (Figure 3 and 4). Similar results for growth of *E. coli* at a temperature of about 19°C in manure rich soils were found by Berry and Miller (2005). Wang et al. (2004) also found greater growth and survival of *E. coli* in dairy cow fecal material at 27°C compared to 4°C or 41°C. Our study with different temperatures was conducted with water but considering the amount of organic matter available from the feces mixed with water this situation can be compared to findings of Berry and Miller (2005).

In the results described above, it can also be observed that at 0°C both the species did not show a statistically significant difference in the *E. coli* concentrations between different days. It was possible because *E. coli* needs at least

7.5°C temperature for growth and is not able to continue protein synthesis below 7.5°C (Shaw et al. 1971). As a result, *E. coli* is growing inconsistently at 0°C showing no significant trends. The *E. coli* concentration in raccoon feces at 10°C showed that *E. coli* can survive for a long time at 10°C. Considering the fact that *E. coli* is a mesophilic organism it was not unexpected for it to show no growth at 50°C, which is too high a temperature for a mesophile to survive. In this study, *E. coli* growth was measured at different temperature conditions using fecal material directly added to water. If *E. coli* isolates from the feces were used instead, different growth results might have been observed. This may be due to the fact that *E. coli* would not have to compete with other bacteria in fecal material. Also, the organic matter availability, as food for bacteria, would have been different under such conditions.

#### Effects of Soil Moisture on Survival of *E. coli*

The growth and survival of *E. coli* under different moisture conditions for cattle and raccoon species showed a similar trend to each other. The maximum survival and growth was observed at 25% moisture content of the soil sample followed by 56.5% moisture content. *E. coli* are facultative anaerobes, which was reaffirmed from the results obtained that the bacteria had the highest growth and survival at 25% moisture content, indicating that among all soil moisture contents selected for this experiment, soil at 25% moisture content provided the most suitable conditions for their survival and growth.

Under dry conditions (4%), bacteria did not totally dieoff, but by 168 h the concentrations reduced considerably; by two orders of magnitude for cattle samples (Figure 5) and by one-half for raccoon (Figure 6) samples. At 56.5% soil moisture content, *E. coli* concentration in cattle showed a gradual increase until 120 h followed by a reduction at 168 h whereas raccoon *E. coli* concentration declined on the fifth day (120 h) and increased on seventh day (168 h). At 83% moisture content, *E. coli* from cattle (Figure 5) reduced after a gradual growth until the fifth day whereas the *E. coli* concentrations in raccoon samples (Figure 6) continued to rise from 1 h to 168 h.

Upon performing the Kruskal-Wallis test on the *E. coli* concentrations obtained in cattle for different moisture contents on different days, it showed that at all four moisture conditions the *E. coli* concentration on each day were different from one another other ( $p < 0.05$ ). The 1 h old samples showed the background concentration of *E. coli* for each moisture condition. A statistical difference in concentration of *E. coli* between different days indicates significant growth or decline. It can be observed that in cattle the 25% moisture condition had the highest *E. coli* concentration on any given day followed by 56.5%, 83%, and

4% moisture contents. The *E. coli* concentration from raccoons samples (Figure 6) showed a similar trend as cattle at 4%, 25% and 83% moisture content ( $p < 0.05$ ) but at 56.5% moisture condition there were no statistical differences in concentrations among different days.

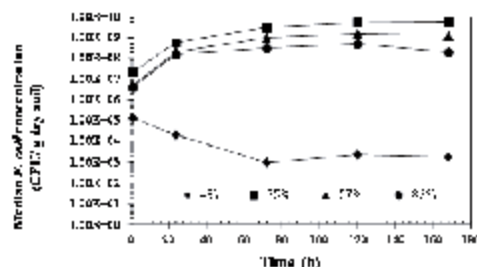


Figure 5 Survival of *E. coli* (isolated from cattle feces) in soil at different moisture contents.

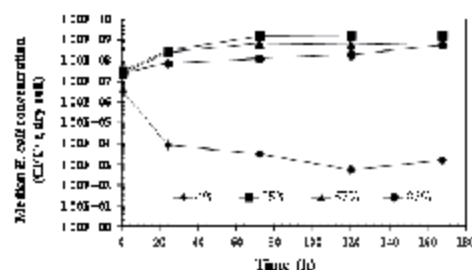


Figure 6 Survival of *E. coli* (isolated from raccoon feces) in soil at different moisture contents.

The test statistics for growth and survival of cattle and raccoons show that *E. coli* concentrations on all days except 1 h ( $p > 0.05$ ) were different for different moisture contents ( $p < 0.05$ ). It was expected for 1 h concentrations to be not significantly different from each other for the different moisture conditions since they are background concentrations. Also, the difference in *E. coli* concentration at different moisture conditions between all the days ( $p < 0.05$ ) suggests growth or decline of *E. coli* after one hour.

Though there is lack of quantitative information on survival rates of enteric bacteria under different soil moisture conditions, numerous studies have suggested that soil moisture is the principal factor affecting the survival of enteric bacteria in soil (Jamieson et al. 2002; Islam et al.

2004; Muirhead et al. 2004, 2006). Chandler and Craven (1978) and Ogden et al. (2001) found a rapid decline in *E. coli* concentration under dry conditions due to desiccation. A study by Jiang et al. (2002) discovered that *E. coli* can continue to exist for extended periods of time at less than 4% moisture condition in soil. We observed that the concentration of *E. coli* from both cattle and raccoons did not die-off at 4% soil moisture within seven days, but the concentrations reduced considerably after 24 h.

In a study by Sjogren (1994) using soil microcosms under controlled conditions in a laboratory, it was found that *E. coli* survived for longer periods under saturated conditions. Hagedorn et al. (1978) and Tate (1978) also found the *E. coli* populations to be greatest under very high moisture conditions in soil. Wang et al. (2004) found interactions between temperature and moisture content. At 27°C, *E. coli* concentrations were greater in dairy cow fecal material at very high (83%) moisture for the first two weeks after excretion, but greater at lower (55% and 30%) moisture thereafter until 15 weeks; however, at 4°C and 41°C, *E. coli* concentrations were consistently greater at very high moisture content for the entire 15-week period. This study found that the survival and growth of *E. coli* peaked at 25% moisture conditions. Chandler and Craven (1978) on the contrary indicated the survival of *E. coli* to be less in soil under cool and moist weather conditions.

The concentration of *E. coli* in this study did increase at 55.6% and 83% moisture but it was less than the concentration found at 25% on any given day. This study was conducted under room temperature conditions. The bacteria possibly found most favorable environment to survive and grow at the particular temperature (room temperature) and 25% soil moisture. Given the facultatively anaerobic nature of these bacteria, it can be assumed that *E. coli* chose to be facultative at 25% soil moisture condition and room temperature as it provided optimum conditions for their survival and growth.

It should be noted that *E. coli* survival and growth in the environment can be influenced by the interacting effects of moisture conditions and temperatures (Wang et al. 2004). Other important physical chemical properties, such as pH, affecting the survival of microorganisms should be taken into account to the study the growth and survival of bacteria. In this study, under different environmental conditions, the temperature and moisture studies were independent of each other. In future studies, interaction of different temperatures and moisture conditions should be considered to study the effect of environment on survival and growth of bacteria. While modeling the fate and transport of *E. coli* in the environment, these complex effects should be considered.

At 20°C temperature and 25% soil moisture content, *E. coli* from both the species seem to show trends similar to each

other, even though there was difference between *E. coli* concentrations of the two species. The kinetic constants for cattle and raccoon *E. coli* concentrations at 20°C temperature (Table 3) and 25% moisture content (Table 4) were similar to each other. It can be observed from the graphs and kinetic constants that at 0°C temperature and at 4% moisture content there was decay (i.e., decline) of *E. coli* concentrations over time. At all other temperatures (except 50°C) and moisture contents, growth was observed. Wang et al. (2004) found *E. coli* in dairy cow fecal material at 83% moisture followed the first-order model only from day 3 to day 20 after excretion. Over this period, *E. coli* exhibited decay with rate coefficients that increased with temperature (-0.0046/h at 4°C, -0.0083 at 27°C, and -0.013 at 41°C). In this study, except at 0°C and 4% moisture content, *E. coli* growth was observed in water and soil. This may be attributed to shorter incubation periods and different *E. coli* isolates used in this study as compared to the study by Wang et al. (2004). Moreover, in this study the isolates were directly added to soil or fecal pellets were added to water. Wang et al. (2004) studied the *E. coli* fate directly in cow manure.

The kinetics analyses in this study show that *E. coli* isolated from cattle feces doubled every 45 hours in water at 10°C while doubling every 16 hours at 20°C. *E. coli* isolates from raccoon feces doubled at a slightly faster rate in water at the same temperature conditions. It would take 38 hours for *E. coli* isolates from cattle to double at 83% moisture condition in soil while it would take roughly 24 hours to double in 25% and 57% moisture condition. It should be noted that *E. coli* isolates from raccoon would double slightly at a slower rate in soil at the same moisture conditions.

**Table 3** First order rate constant for *E. coli* survival in water.

	$k_T (\text{hr}^{-1})$	
	Cattle	Raccoon
0 °C	-0.0025	-0.0073
10 °C	0.0151	0.0218
20 °C	0.0425	0.0472

**Table 4** First order rate constant for *E. coli* survival in soil.

	$k_{MC} (\text{hr}^{-1})$	
	Cattle	Raccoon
4%	-0.0237	-0.0385
25%	0.0289	0.0207
55.6%	0.0281	0.0162
83%	0.0182	0.0162

## CONCLUSIONS

Four different non-point sources of *E. coli* were identified in Cedar Creek watershed. The sources were quantified for their *E. coli* content. *E. coli* concentrations were reported as CFU/g of dry fecal material. Cattle showed variability in *E. coli* concentrations between adult and calves, with calves having higher *E. coli* concentration in their feces than adults. No statistical differences in fecal *E. coli* concentrations were detected between males and female for any species.

The growth and survival of *E. coli* subjected to different temperature conditions showed high variability in results over time. *E. coli* concentrations in cattle and raccoon feces showed the highest survivability and growth at 20°C out of all the temperatures studied. There was no survival of *E. coli* from either species at 50°C after 24 h. *E. coli* in cattle and raccoons samples exhibited greater growth at lower, nearly aerobic soil moisture content (25%) for all days compared to nearly anaerobic soil moisture content (83%).

This study verified the facultative behavior of *E. coli* contributing to accelerated growth levels at cooler temperature and nearly aerobic conditions. Future studies should consider the effect of the interaction of different temperatures and moisture conditions on the survival and growth of *E. coli* in animal feces.

Watershed modeling tools generally lack the capacity to simulate bacteria life cycle and behavior under different environmental conditions. The growth trends observed under different environmental conditions in this study would improve prediction of *E. coli* loads in a waterbody during different times of a year, thus helping to address seasonal variation, which is one of the major factors governing the bacterial loadings in a water body. Understanding the behavior of bacteria under different environmental conditions also helps to develop proper manure management techniques before land application of manure.

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## **Appendix B:**

### **“Growth Kinetics of Wildlife *E. coli* Isolates in Soil and Water”**

## Growth Kinetics of Wildlife *E. coli* Isolates in Soil and Water

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### ABSTRACT

Bacteria are the major cause of surface water contamination in the United States. US Environmental Protection Agency (USEPA) uses the Total Maximum Daily Load (TMDL) process to regulate the *E. coli* loads from fecal sources in a watershed. Different point and non-point sources can contribute to the fecal contamination of a waterbody including municipal and on-site wastewater treatment plants, livestock, birds, and wildlife. Unfortunately, wildlife sources in many rural watersheds are poorly characterized. *E. coli* is also known to persist in waterbodies when no known fecal sources are present. In this study, *E. coli* from wildlife fecal material was enumerated and the fate of *E. coli* under different environmental factors was studied. No growth was observed in soil at 4% moisture content and in water at 10°C. The highest *E. coli* growth was recorded in water at 30°C. It can be seen from these results that there was variation in the fate of *E. coli* under different environmental conditions. The fate of *E. coli* in the environment is a complex process and is influenced by many factors and their interactions, making it difficult to predict. The findings from this study along with additional studies can be used to improve the accuracy of model predictions to estimate the *E. coli* loads in watersheds.

**Keywords:** Bacteria; Fecal Contamination; TMDL; Water Quality

### 1. Introduction

The leading cause of impairment for waterbodies in the United States is from bacteria [1]. Bacterial impairment of rivers and streams originates from fecal contamination. Wastewater effluents and fecal material from both livestock and wildlife are potential sources of fecal contamination in a watershed. Warm-blooded mammals shed pathogenic bacteria in their feces. Pathogenic bacteria such as *Salmonella typhi*, *Shigella*, *Campylobacter jejuni*, and *Escherichia coli* (*E. coli*) O157:H7 are responsible for waterborne diseases that include typhoid fever, dysentery, campylobacteriosis, and *E. coli* O157:H7 infection, respectively. These illnesses can include symptoms of diarrhea, fever, nausea, vomiting, and abdominal cramps. Few of these symptoms can last for days and even lead to death in immune-compromised individuals [2]. *E. coli* is the current indicator organism for fecal contamination and used by the Environmental Protection Agency (EPA) to assess bacterial impairment in waterbodies. In 2008, 405 streams in Texas were bacterially impaired according to the 303(d) list of impaired waterbodies in the United States [3].

Under sub-tropical and temperate environments *E. coli* has been observed to persist [4-8]. Sources of *E. coli* in a waterbody are not only external sources but also *in situ*. Specifically, sediments have been found to be reservoirs of enteric bacteria, including *E. coli*, and a potential source in waterbodies [4-13]. Environmental controls have been shown to have a role in sustaining *E. coli* populations in the environment [14-16].

The trends of *E. coli* survival in variety of waterbodies including lake, river, sea, and creek have been studied [17-24]. Temperature is suggested to be the most important factor that affects bacterial survival in water [18]. The survival and growth rate of *E. coli* in river water can be affected by temperature differently. Hendricks (1972) observed a higher growth rate of *E. coli* in river water at 30°C than at lower temperatures. However, Flint (1987) observed the survival of *E. coli* in river water was less at 30°C than at 4°C and 25°C. Filip *et al.* (1988) also observed longer survival of *E. coli* at lower temperatures. They reported *E. coli* survived for 100 days in ground-water at 10°C [19]. Additionally, Padia (2010) found that *E. coli* survival in creek water was the highest at 20°C compared to *E. coli* survival at 0°C, 10°C, and 50°C over a period of one-week.

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Growth and survival of *E. coli* in soil is affected by moisture content [6,8,14-16,25,26]. Change in soil moisture content from dry to saturated conditions was found to promote the growth of *E. coli* in soil [6,8]. At dry soil conditions, the *E. coli* die-off was observed to be faster than saturated soil moisture conditions [25]. Sjogren (1994) set-up laboratory soil microcosms and observed that the survival of *E. coli* was the longest when soil was under saturated moisture conditions, lasting up to 23.3 months.

Water quality and watershed modeling tools such as Spatially Explicit Load Enrichment Calculation Tool (SELECT), Hydrological Simulation Program-Fortran (HSPF), and Soil and Water Assessment Tool (SWAT) are used in the bacterial Total Maximum Daily Load (TMDL) process to characterize *E. coli* sources in a watershed and estimate the required reductions in *E. coli* loads [27-30]. In rural watersheds, wildlife can contribute a majority of the fecal pollution and should be considered [31]. Unfortunately, *E. coli* concentrations present in wildlife fecal material are not well documented. Watershed modeling tools incorporate environmental factors to estimate *E. coli* loads in a watershed but more data are needed [32].

The main objective of this research was to study the fate of *E. coli* isolates from wildlife fecal material in water at different temperatures and in soil at different moisture conditions. The specific objectives were to 1) enu-

merate and obtain isolates from wildlife species' fecal material; 2) determine kinetic characteristics of *E. coli* isolates enumerated from feral hog and deer fecal material in water at different temperatures; and 3) determine kinetic characteristics of *E. coli* isolates enumerated from feral hog and deer fecal material in soil at different soil moisture conditions.

## 2. Study Area

Cedar Creek watershed is located in East Central Texas, USA, within both Brazos and Robertson County (Figure 1).

Cedar Creek is one of the 405 impaired water bodies in Texas that does not meet the bacteria criteria for the state [3]. It also is categorized as 5c which requires additional data and information for a TMDL to be scheduled by the Texas Commission on Environmental Quality (TCEQ). There is very little urban influence in Cedar Creek. The land use is mainly rangelands and forested areas (Table 1). Direct fecal deposition from cattle, wildlife, and birds along with other non-point sources contribute to the fecal contamination of the creek.

## 3. Methodology

### 3.1. Sampling Protocol

Two sub-watersheds within Cedar Creek watershed were

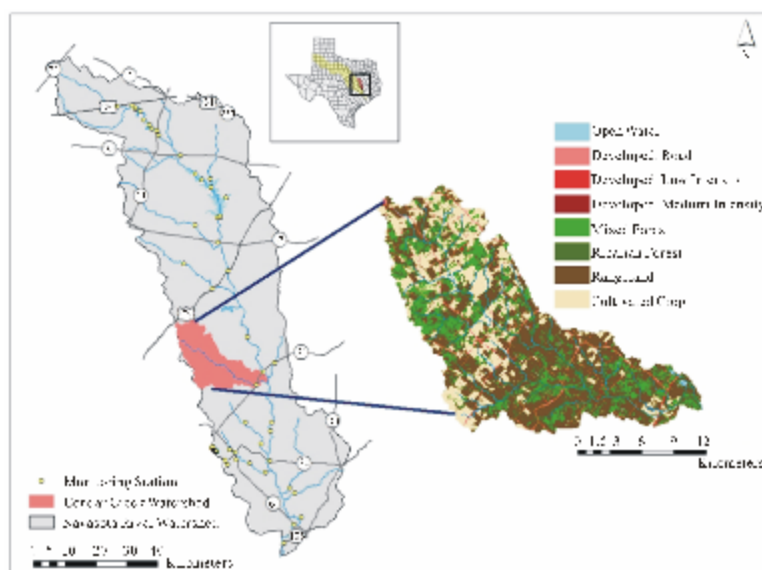


Figure 1. Location of Cedar Creek Watershed in central Texas.



Table 1. Cedar creek watershed characteristics.

Total Area	340.54 km <sup>2</sup>
Land Use	95.3% undeveloped forest 3.9% developed area 0.82% open waters
Climate	subtropical
Rainfall (Annual)	810 - 1220 mm
Soil*	sandy loam (66% sand, 18% silt, and 16% clay) 1.2% organic matter strongly acidic (pH 5.2)

used for sampling with landowner co-operation. The land use of the sub-watersheds is mainly rangeland. The sampling protocol for obtaining the fecal material included a grid-design for trapping the wildlife. A wildlife expert designed the protocol for trapping and collecting the fecal material. The wildlife species trapped included raccoon, opossum, feral hog, deer, skunk, and armadillo. A more detailed description of sampling protocol is discussed in [24]. Briefly, once the wildlife species were trapped the fecal material was collected using sterile Whirl-Pak® bags while wearing latex gloves. The sex, age, date of trapping, and location were recorded. The samples were transported in an insulated cooler on ice at 5°C to the Water Quality Engineering Laboratory at Texas A & M University.

### 3.2. Isolating *E. coli* from Wildlife Fecal Samples

The fecal samples were brought to the lab and kept frozen at -20°C until processed. When processed, the fecal material was defrosted and one gram was measured out with sterile scoop. The samples were serially diluted in de-ionized (DI) water. The diluted samples were run through a membrane filtration system, following the EPA method 1603 [33]. The membrane of 0.45 µm pore size was removed from the filtration system with sterile forceps and placed on modified Thermo-tolerant *E. coli*, mTEC, (Difco®) agar plates. The plates were inverted and placed in an incubator at 35.5°C ± 0.5°C for two hours to revive the cells. Then the plates were sealed in Whirl-Pak® bags and placed in a water bath at 44.5°C ± 0.5°C for 22 hrs to select for thermo-tolerant *E. coli*. The plates were counted and values of 30 to 300 CFUs (colony forming units) were recorded.

Randomly selected isolates from each plate were streaked on Nutrient agar with, 4-methylumbelliferyl-β-D-glucuronide (MUG), (Difco®) and placed inverted in an incubator at 35.5°C for 24 hrs. MUG is a colorless substrate that is hydrolyzed by an enzyme present in *E. coli*, to a fluorescent product, 4-methylumbelliferone. *E. coli* was confirmed if the MUG plates fluoresced. Confirmed isolates were stored in labeled centrifuge tubes

with 1 mL of Luria-Bertani (LB) broth (Difco®) and 10% glycerol in -20°C freezer.

### 3.3. Growth Kinetics of *E. coli* in Water under Different Temperatures

Three *E. coli* isolates from each feral hog (H1-3, H4-1, and H7-1) and deer (D1-c, D2-c, and D2-d) fecal sample were enriched in 100 mL of LB broth at 35.5°C for 24 hrs. The enriched LB broth was diluted to 10<sup>-4</sup> by adding 1 mL of LB broth into 100 mL sterile DI water, stirred, and then 1 mL of the diluted LB broth was added to 100 mL of sterile DI water. Finally, creek water microcosms were made with 1 mL of the 10<sup>-4</sup> dilution of LB broth is added to 100 mL of sterile Cedar Creek water (autoclaved three times at 121°C for 15 min). The creek water microcosms were triplicated and kept in an incubator set according to the experimental temperatures (10°C, 25°C, and 30°C). Over 30 hrs, 0.1 mL of each water microcosm were spread plated onto MacConkey agar (Difco®) plate at different sampling times. The *E. coli* concentrations (CFU/mL) were recorded at each sampling time.

The kinetic characteristics of *E. coli* strains in water at different temperatures were determined using first order kinetics. The natural log of the bacterial counts was plotted against time to obtain the rate constant, k. A trend line was fitted to the data to determine the k-value from the slope of the line. If the k-value was positive, then the doubling time (t<sub>d</sub>) was calculated. If the k-value was negative, then the half-life (t<sub>1/2</sub>) was calculated.

### 3.4. Growth Kinetics of *E. coli* in Soil under Different Moisture Conditions

One *E. coli* isolate from each feral hog (H1-3) and deer (D2-c) fecal sample was enriched in 100 mL of LB broth. Cedar Creek soil (Table 1; autoclaved three times at 121°C for 15 min and dried in oven for 10 hrs) was used in soil microcosms with 1 mL of enriched LB broth. An estimated amount of sterile DI water (0, 6, and 15 mL) was added to 30 g of soil to obtain experimental moisture contents (4%, 25%, and 57%). Three replicated soil microcosms were placed on a rotary shaker at 200 rpm at room temperature (22°C). Over one week, *E. coli* was enumerated by serially diluting one gram soil from each microcosm in DI water and 0.1 mL of the last three dilutions was spread-plated onto MacConkey agar plates. The *E. coli* bacterial counts (CFU/g) were recorded at five to seven different sampling times for the duration of a week. One isolate from each MacConkey plate with growth was randomly selected and streaked onto nutrient agar with MUG and grown at 35.5°C for 24 hrs to confirm the isolate was still *E. coli* and not contamination.

The kinetic characteristics of *E. coli* strains in soil at different soil moisture conditions were determined in the

same manner as described in Section C.

## 4. Results and Discussion

### 4.1. Growth of *E. coli* in Water at Different Temperatures

The change in concentration of *E. coli* isolates from feral hog and deer fecal material in sterilized Cedar Creek water was observed at different temperatures. Preliminary eight hour studies were conducted and it was found that water temperature affected the *E. coli* concentration over time. Concentration of *E. coli* increased in water at 25°C and at 30°C over time. At 10°C, decrease in *E. coli* concentration was observed (data not shown).

These findings were further tested with one isolate from feral hog fecal material (H1-3) and one isolate from deer fecal material (D2-c) over 30 hours. *E. coli* concentrations for H1-3 decreased in water at 10°C and increased in water at 25°C and 30°C (Figure 2). The change in *E. coli* concentrations for D2-c was the same for all three temperatures (Figure 2). Padia (2010) and Hendricks *et al.* (1972) observed increase in *E. coli* concentration in water at 30°C over time. However, [34] and [35] reported *E. coli* concentrations in water decreased at higher temperatures, such as 30°C.

These contradicting observations in the fate of *E. coli* in water at 30°C might be due to the difference in *E. coli* isolates used in these studies. In this study and the study by [24], thermo-tolerant *E. coli* was enumerated directly from the fecal material of potential sources, whereas laboratory *E. coli* strains that did not originate from fecal material were used in other studies [34,35]. The high temperature of the intestinal tract of mammals (~30°C) promotes growth of thermo-tolerant enteric bacteria, such as *E. coli*. For this reason, the EPA method to enumerate *E. coli* in water uses a temperature of 44.5°C to select for thermo-tolerant *E. coli* [33]. The results from this study using thermo-tolerant *E. coli* give a more accurate depiction of the fate of *E. coli* from fecal deposition compared to studies using laboratory *E. coli* strains.

The kinetic constant (*k*), doubling time (*t<sub>d</sub>*), and half-life (*t<sub>1/2</sub>*) were determined assuming first order kinetics.

The kinetic characteristics for *E. coli* isolates, H1-3 and D2-c, at each water temperature are shown in Table 2. The kinetic constants for both H1-3 and D2-c were the lowest at 10°C and the highest at 30°C in water (Table 2). The kinetic constants for H1-3 and D2-c were negative, indicating the *k*-value was a decay rate (Table 2). The growth rate of *E. coli* varies with temperature and is crucial in determining total maximum daily loads (TMDLs) for *E. coli*. The duration of this study was 30 hours in the laboratory but considered a day, 24 hours, in the aquatic environment. The doubling time/half-life, *t<sub>d</sub>*/*t<sub>1/2</sub>*, along with the *k*-value, can be used to quantify the amount of *E. coli* present after a period of time.

### 4.2. Growth of *E. coli* in Soil under Different Moisture Conditions

Concentrations of *E. coli* isolates from feral hog and deer fecal material in sterilized Cedar Creek soil (Table 1) at different moisture conditions were measured over time (Figure 3). Both *E. coli* isolates from feral hog (H1-3) and deer (D2-c) decreased in concentrations over 168 hours for 4%, 25%, and 57% moisture content, with the exception of H1-3 at 57% with a slight increase in concentration. At 4% moisture content, the *E. coli* concentrations for both isolates (H1-3 and D2-c) decreased to zero after only 50 hours. These observations were explained by first order kinetic constant (*k*), doubling time (*t<sub>d</sub>*), and half-life (*t<sub>1/2</sub>*). Kinetic study results for *E. coli* isolates, H1-3 and D2-c, in soil at different moisture contents are presented in Table 3.

In this study, a greater rate of decay was observed for *E. coli* in soil at 4% moisture content compared to soil at 25% or 57% moisture content (Table 3). Previous studies reported higher die-off rate for *E. coli* in dry soil than in saturated soil [14,15,25,36]. Based on the results from this study, *E. coli* concentration in dry soil with 4% moisture content will decrease to half the initial concentration after approximately three to four hours (Table 3). This rapid decay of *E. coli* concentration in dry soil would continue to occur until the *E. coli* concentrations becomes zero.

Table 2. Kinetic characteristics of *E. coli* isolates from feral hog and deer feces in sterilized Cedar Creek water at different temperatures.

Temperature	Feral hog (H1-3)			Deer (D2-c)		
	<i>k</i> (hr <sup>-1</sup> )	<i>t<sub>1/2</sub></i> (hr)	<i>t<sub>d</sub></i> (hr)	<i>k</i> (hr <sup>-1</sup> )	<i>t<sub>1/2</sub></i> (hr)	<i>t<sub>d</sub></i> (hr)
10°C	-0.0261 ( <i>R</i> <sup>2</sup> = 0.89)	26.6	-	-0.0286 ( <i>R</i> <sup>2</sup> = 0.35)	24.2	-
25°C	0.0812 ( <i>R</i> <sup>2</sup> = 0.86)	-	8.5	0.0985 ( <i>R</i> <sup>2</sup> = 0.86)	-	7.0
30°C	0.109 ( <i>R</i> <sup>2</sup> = 0.91)	-	6.4	0.198 ( <i>R</i> <sup>2</sup> = 0.97)	-	3.5

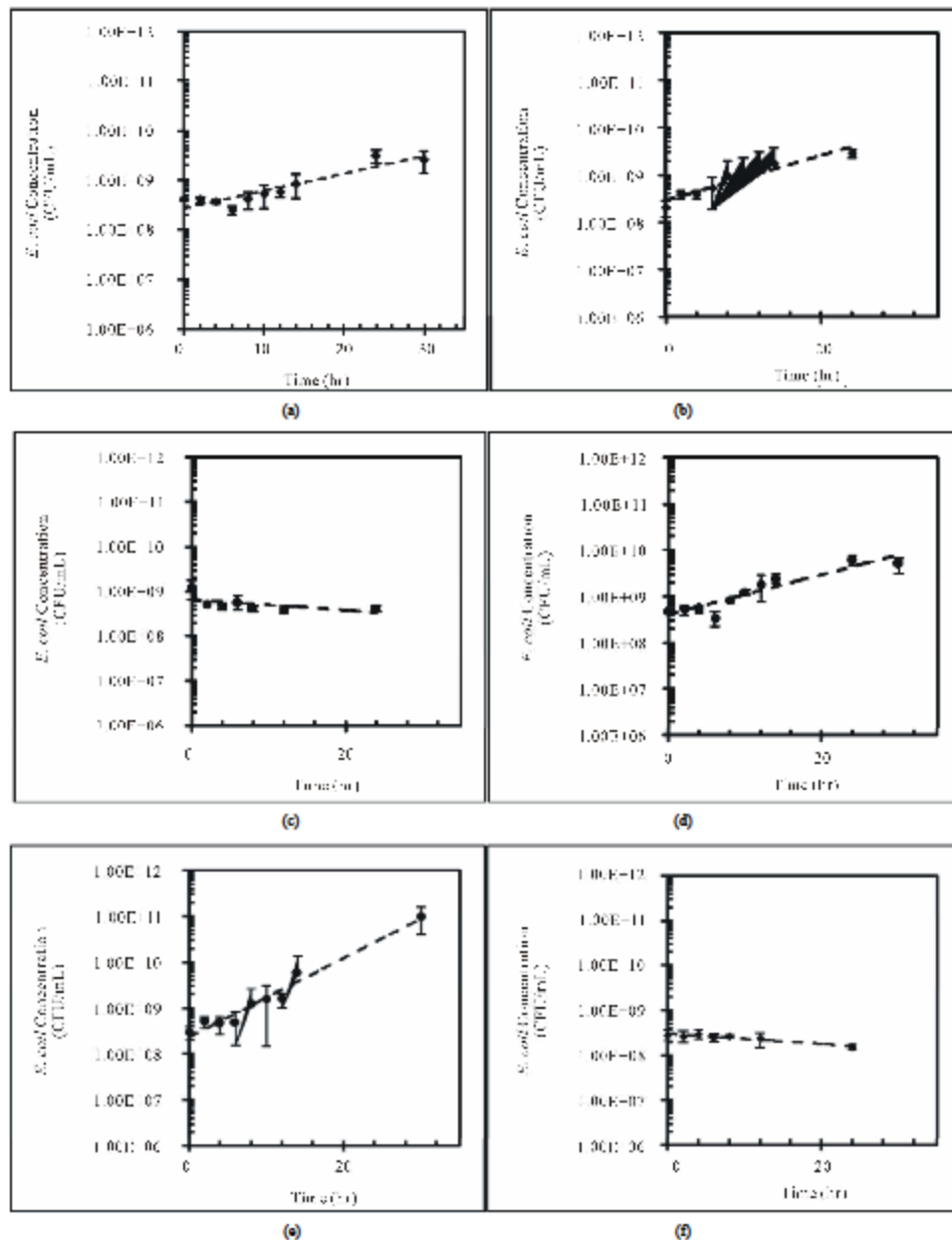


Figure 2. Concentrations (CFU/mL) of *E. coli* isolates, H1-3 and D2-c, in sterilized Cedar Creek water at different temperatures over time, (a) H1-3 at 10°C; (b) H1-3 at 25°C; (c) H1-3 at 30°C; (d) D2-c at 10°C; (e) D2-c at 25°C; (f) D2-c at 30°C.

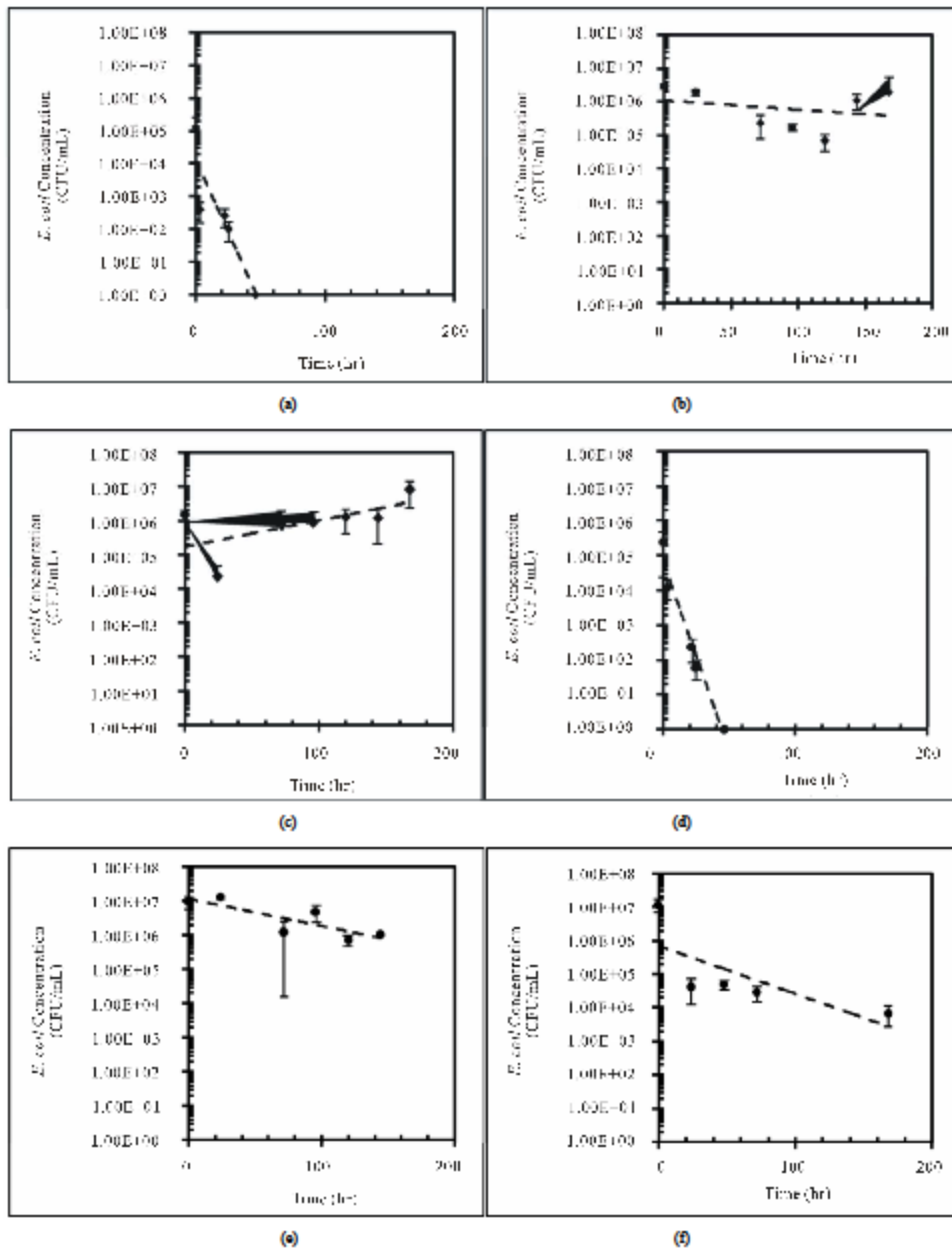


Figure 3. Concentrations (CFU/mL) of *E. coli* isolates, H1-3 and D2-c, in sterilized Cedar Creek soil at different moisture content over time, (a) H1-3 at 4%; (b) H1-3 at 25%; (c) H1-3 at 57%; (d) D2-c at 4%; (e) D2-c at 25% (f) D2-c at 57%.

**Table 3.** Kinetic characteristics of *E. coli* isolates from feral hog and deer feces in sterilized Cedar Creek soil at different moisture contents.

Volumetric Moisture Content	Feral hog (H1-3)			Deer (D2-c)		
	k (hr <sup>-1</sup> )	t <sub>0.5</sub> (hr)	t <sub>d</sub> (hr)	k (hr <sup>-1</sup> )	t <sub>0.5</sub> (hr)	t <sub>d</sub> (hr)
4%	-0.2016 (R <sup>2</sup> = 0.81)	3.4	-	-0.2539 (R <sup>2</sup> = 0.96)	2.7	-
25%	-0.0065 (R <sup>2</sup> = 0.08)	106.6	-	-0.0187 (R <sup>2</sup> = 0.69)	37.1	-
57%	0.0177 (R <sup>2</sup> = 0.38)	-	39.2	-0.0326 (R <sup>2</sup> = 0.55)	21.3	-

Several previous studies showed *E. coli* growth in saturated soils [14,15,25]. At 57% soil moisture content, growth was observed in *E. coli* isolated from feral hog feces (H1-3) while decay was observed in *E. coli* isolated from deer feces (D2-c). This difference may be due to the difference in moisture content of feral hog and deer fecal material. While processing the samples, it was observed that feral hog feces had more moisture than deer feces. Previous study showed the die-off of *E. coli* varied with the moisture condition of the fecal material [37].

It should be noted that in this study the effect of soil moisture content on the growth of *E. coli* was conducted in sterile soil microcosms, keeping all other environmental variables constant. Non-sterile soil might have resulted in lower *E. coli* concentrations due to competition for nutrients by the other microorganisms present in the soil [11,38]. The level of competition from other microorganisms is variable from soil to soil and would be difficult to predict. This study was designed to gain understanding of the fate of *E. coli* under different moisture contents with little to no competition within the soil.

## 5. Conclusions

Six different wildlife sources that could potentially contribute to *E. coli* contamination in Cedar Creek watershed, Texas were identified. The wildlife sources included feces from raccoon, opossum, feral hog, deer, skunk, and armadillo. The fecal material of each wildlife species was enumerated for *E. coli* and reported as CFU per g wet basis. The large range and variability in *E. coli* concentrations among few of the wildlife feces could be because of diverse dietary habits. There was a statistically significant difference in *E. coli* concentration in fecal samples collected in the summer and winter.

The growth of *E. coli* in sterilized Cedar Creek water at different temperatures varied depending on water temperature. Both deer and feral hog fecal *E. coli* isolates had the highest growth rate in water at 30°C and both isolates died off in water at 10°C. At 4% soil moisture content, both isolates died off rapidly. This suggests that under dry soil conditions *E. coli* will not survive for a

longer duration and will not pose a threat to nearby waterbodies. The *E. coli* isolate from feral hog fecal sample persisted in soil at 25% moisture content and slightly grew at 57% moisture content. However it should be noted that the correlation between *E. coli* concentration and these moisture contents was very low. Results from this study show the high variability in *E. coli* persistence, survival, and decay in terrestrial environment. It is evident that fate of *E. coli* in the environment is a complex process and governed by various factors. The interacting effects of these environmental factors add varying degrees of complexity to model and predict fecal contamination in watersheds.

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